Interaction between Hormone and Apoplastic ROS Signaling in Regulation of Defense Responses and Cell Death

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“*Try not to become a man of success but rather try to become a man of value*”—*Albert Einstein*.

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Original publications

This thesis is based on the following original publications. The publications are referred to in the text by Roman numerals

Publications and manuscripts:

I. Xu, E., & Brosché, M. (2014). Salicylic acid signaling inhibits apoplastic reactive oxygen species signaling. *BMC plant biology*, 14(1), 155.

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III. Xu, E[1]., Vaahtera, L.[1], & Brosché, M. (2015). A transcriptome analysis of apoplastic reactive oxygen species signaling in Arabidopsis and dissection of its regulatory pathways. Manuscript. [1] Shared first author.

Author's contribution:

I. EX participated in experimental design, performed phenotypic and genotypic analysis of double and triple mutant, gene expression studies, quantification of cell death, performed data analysis, and wrote the manuscript.

II. EX participated in experimental design, crossed C24 with Te, C24 with CT101 to generate F2 population, implemented all phenotyping of O_3 treatment, genotyping of F2 populations O_3 treatment, SA treatment, quantification of cell death and visualization of H_2O_2, RNA-seq samples preparation, involved in RNA-seq data analysis, performed QTL, Microarray and statistics analysis and wrote the manuscript.

III. EX participated in experimental design, performed Microarray array analysis, qPCR gene expression analysis, involved in RNA-seq sample collection, quantification of cell death and visualization of H_2O_2, and participated in writing the manuscript.

Abbreviations

ABA	Abscisic acid	MAMP	Microbe-associated pattern
ABI	ABA insensitive	MAPK	Mitogen-activated protein kinases
ACS	ACC synthase	MeJA	Methyl-jasmonic acid
		NAC	NAM, ATAF1, 2, and CUC2
APX	Ascorbate peroxidase	NPR	Nonexpresser of PR gene
BIK1	BOTRYTIS-INDUCED KINASE1	NUDX	*NUDIX HYDROLASE HOMOLOG*
BTH	Benzothiadiazole	$^1O_2$	Singlet oxygen
Ca^{2+}	Calcium ion	$O_2^{\bullet-}$	Superoxide anion
CaMs	Calmodulin proteins	O_3	Ozone
CAT	Catalases	OPDA	Cyclopentenone cis-(+)-12-oxophytodienoic acid
CBLs	Calcineurin B-like proteins	OST1	OPEN STOMATA 1
CDPKs	Ca^{2+}-dependent protein kinases	PAL	Phenylalanine ammonia-lyase
CIM	Constitutive immunity	PAMP	Pathogen-associated pattern
CIPKs	CBL-interacting protein kinases	PCD	Programed cell death
CMLs	Calmodulin-like proteins	PM	Plasma membrane
CNGC	Cyclic nucleotide gated-ion channels	PP2Cs	Type 2C protein phosphates
CPR	Constitutive expressor of PR genes	PR	Pathogenesis-related gene
DAMP	Damage-associated molecular pattern	PRX	PEROXIDASE
DPI	Diphenyliodonium		
DREBs	Dehydration-Responsive Element Binding-proteins	PYR/PYL/RCAR	PYRABACTIN RESISTANCE/PYR1 LIKE/REGULATORY COMPONENT OF ABA RECEPTOR
ERF	Ethylene-responsive element binding factor	qPCR	Real time reverse transcriptase quantitative PCR
ETC	Mitochondrial electron transport chain	QTL	Quantitative trait loci
flg22	Flagellin peptide	RBOHs	Respiratory burst oxidase homologs
FLS2	FLAGELLIN-SENSITIVE 2	RIL	Recombinant inbred line
GLR	Glutamate receptor-like	RLK	Receptor-like kinases
GO	Gene ontology	RNA-seq	RNA sequencing
H_2O_2	Hydrogen peroxide	RNAi	RNA interference
HO•	Hydroxyl radical	ROS	Reactive oxygen species
HR	Hypersensitive response	SA	Salicylic acid
ICS	Isochorismate synthase	SLAC1	SLOW ANION CHANNEL-ASSOCIATED 1
JA	Jasmonic acid	SNP	Single nucleotide polymorphism
K^+	Potassium ion	SnRK2	SNF-related kinases
LMM	Lesion mimic mutant	SOD	Superoxide dismutase
LRR	Leucine-rich repeats	TFs	Transcription factors
LSD	Lesion simulating disease		

Abstract

Regulation of cellular homeostasis is crucial for proper development, survival, defense responses, programmed cell death and ultimately survival. Maintaining cellular homeostasis requires tight regulation of multiple highly interactive signaling pathways. The apoplast lies at the frontier between the cell and the environment, where the plant perceives environmental cues. Since the apoplast is also a site for cell-to-cell communication, it has an important role in mediating plant-environment interactions. Reactive oxygen species (ROS) are known as both toxic agents and indispensable signaling molecules in all aerobic organisms. A ROS burst in the apoplast is one of the first measurable events produced in response to different biotic and abiotic stresses, eventually leading to the initiation of signal transduction pathways and altered gene expression. Apoplastic ROS signaling is well known to dynamically coordinate multiple signaling pathways in the activation of defense responses in plants. Dissection of the signaling crosstalk within such a signaling network could therefore reveal the molecular mechanisms underlying defense responses. Treatments with ozone (O_3) have been adopted as an efficient tool to study apoplastic ROS signaling. Plants exposed to O_3 trigger a ROS burst in the apoplast and induce extensive changes in gene expression and alteration of defense hormones, such as salicylic acid (SA), jasmonic acid (JA), and ethylene.

Genetic variation in O_3 sensitivity among *Arabidopsis thaliana* accessions or mutants highlights the complex genetic architecture of plant responses to ROS. To gain insight into the genetic basis of apoplastic ROS signaling, a recombinant inbred line (RIL) population from a reciprocal cross between two Arabidopsis accessions C24 (O_3 tolerant) and Tenela (O_3 sensitive) was used for quantitative trait loci (QTL) mapping. Through a combination of QTL mapping and transcriptomic analyses in the response to apoplastic-ROS treatment, three QTL regions containing several potential candidate genes were identified in this study. In addition, multiple mutants with varying O_3-sensitivities were employed to dissect the signaling components involved in the early apoplastic ROS signaling and O_3-triggered cell death. A combination of global and targeted gene expression profiling, genetic analysis, and cell death assays was performed to dissect the contribution of hormone signaling and various transcription factors to the regulation of apoplastic ROS-triggered gene expression and cell death.

The contributions of SA, JA and ethylene were assessed through analysis of mutants deficient in these hormones, mutants with constitutively activated hormone signaling and the exogenous application of hormones. Plants with elevated SA levels were found to be associated with an attenuated O_3 response, whereas simultaneous elimination of SA-dependent and SA-independent signaling components enhanced the response to apoplastic ROS treatment. JA could act as both a positive and negative modifier of apoplastic ROS signaling, which was enhanced when ethylene signaling was also impaired. However, transcriptome analysis of a triple mutant deficient in SA, JA and ethylene revealed that these hormones signaling only contributed part (about 30%) of early-apoplastic ROS-triggered changes in gene expression, suggesting multiple signaling pathways could be required to regulate the apoplastic ROS response via combinatorial or overlapping mechanisms.

1 Introduction

Reactive oxygen species (ROS) signaling networks are used in all aerobic organism and regulate a broad range of physiological responses, such as growth, development, and responses to biotic and abiotic stresses (Foreman et al., 2003; Gapper and Dolan, 2006; Baxter et al., 2014; Schieber and Chandel, 2014). ROS are formed upon the incomplete reduction of oxygen, including superoxide anion ($O_2^{\bullet-}$), hydrogen peroxide (H_2O_2), and the hydroxyl radical (HO•) as well as other ROS, singlet oxygen ($^1O_2$) and ozone (O_3) (Demidchik, 2015). The study of ROS burgeoned over a century ago (Nathan and Cunningham-Bussel, 2013), and ROS were long regarded as unwanted and toxic compounds in physiological metabolism. However, during the last two decades our understanding of the role of ROS has largely expanded from them merely being harmful species causing oxidative stress to the view that they are essential messengers and involved in redox signaling. The sessile nature of plants necessitates their adaptation to the ever-changing environment. As such, plants have evolved elaborate signaling systems including an oxidative burst to alter metabolism and to mount effective defenses against biotic and abiotic stresses. This response involves the spatiotemporal production of a ROS burst in the intra and extracellular space, changes in concentration of cytosolic-free calcium $[Ca^{2+}]_{cyt}$, activation of signaling cascades, transcriptome reprogramming and altered production of hormones (Vaahtera and Brosché, 2011; Pieterse et al., 2012; Steinhorst and Kudla, 2013). The interaction between these signaling pathways and ROS production allows precise modulation of plant growth and defense in response to various environmental stimuli.

Apart from exogenous sources of ROS (for example via O_3 or ROS produced from high energy UV-B radiation), plants produce significant amounts of ROS in several intracellular compartments (the chloroplast, peroxisome and mitochondrial) as a result of photosynthesis, photorespiration, respiration and other metabolism (Das et al., 2015). Such pathways contribute to the control of redox-regulated signaling within and between different organelles and relay the information to the nucleus to regulate gene expression (Sierla et al., 2013; Vaahtera et al., 2014). Like the intracellular compartments, the apoplast makes a substantial contribution to ROS production in response to biotic and abiotic stresses. The apoplast is a space outside of the plasma membrane (PM), hosting a number of activities including signal recognition, cell-to-cell communication and pathogen defenses (Daudi et al., 2012; Steinhorst and Kudla, 2013; Gilroy et al., 2014). An apoplastic ROS burst induced by extracellular stimuli is one of the earliest events in plant defense responses. Receptors or ion channels on the PM can sense this burst and transduce it through cytosolic signaling, activation of cell-to-cell communication and formation of a ROS wave that can carry such signals across different tissues (Wrzaczek et al., 2010; Steinhorst and Kudla, 2013; Wrzaczek et al., 2013; Kadota et al., 2014).

At another level of regulation, membrane localized or associated proteins such as heterotrimeric G-proteins and NADPH oxidases (respiratory burst oxidase homologs, RBOHs) are crucial components connecting extra- and intra-cellular ROS signaling (Joo et al., 2005; Torres et al., 2005). Ion channels are proteins that form hydrophilic pathways across all plant membranes. Accumulating evidence indicate that increased anion channel activity is directly involved in the control of stomatal movement and other events involving oxidative stress such as programed cell death (PCD) (Kadono et al., 2010). Likewise, perception of microbe- or

pathogen or damage-associated molecular patterns (MAMPs, PAMPs or DAMPs) by receptor-like kinases (RLK) at the PM also trigger production of apoplastic ROS via phosphorylation and activation of NADPH oxidases RBOHD (Ranf et al., 2011; Osakabe et al., 2013; Idänheimo et al., 2014; Kadota et al., 2014). Subsequently, intracellular signal transduction and PCD are modulated by a dynamic interaction of multiple components including ROS, $[Ca^{2+}]_{cyt}$, hormone signaling (salicylic acid, SA; jasmonic acid, JA; ethylene; abscisic acid, ABA), mitogen-activated protein kinases (MAPK) signaling cascades and antioxidants. Clearly *de novo* ROS biosynthesis in response to biotic and abiotic stresses is among most important components of stress signaling and immunity responses in plant.

The introductory part of this thesis aims to give a short summary of the role of ROS in different aspects of signaling and cell physiology: i.e. The role of ROS signaling and other signaling in the defense mechanism; the role of hormones in apoplastic ROS-induced cell death and defense responses and the role of transcription factors in apoplastic ROS signaling. This thesis work focused on dissection of the signaling components involved in the apoplastic ROS signaling. A combination of genetic analysis and transcriptome analysis was employed to quantify the contribution of hormone signaling and various transcription factors (TFs) to the regulation of gene expression and cell death.

1.1 Rapid activation of defense mechanisms in plants

The cellular homeostasis in plants is constantly changing due to pathogens and environmental fluctuations, and therefore sensitive mechanisms must have evolved to allow rapid perception of environmental cues and concomitant modification of growth and defense for adaptation and survival. The rapid production of ROS, together with altered ion fluxes, activation of MAPK kinase cascades and hormone-signaling in response to stress are generally considered a defense mechanism for resistance against microbes, initiation of defense response and regulation of PCD in plants (Suzuki et al., 2014).

1.1.1 ROS production in the apoplast in response to stress

Production of ROS in the apoplast is one of the first measurable events shared among different biotic and abiotic stresses (Wojtaszek, 1997). Mounting evidence indicates that rapid accumulation of apoplastic ROS during biotic and abiotic stresses is mediated by the activities of two types of enzymes: NADPH oxidases and class III cell wall peroxidases (Daudi et al., 2012; O'Brien et al., 2012). Plant NADPH oxidases (NOXs) known as RBOHs (respiratory burst oxidases), are enzyme-complexes localized on the PM; RBOHs utilize NADPH as a cytosolic electron donor to reduce extracellular O_2 to $O_2^{\bullet-}$, which subsequently undergoes superoxide dismutase (SOD)-catalyzed disproportionation to O_2 and H_2O_2. Class III cell wall peroxidases, on the other hand, could form H_2O_2 without the activity of SOD (Almagro et al., 2009).

RBOHs are integral plasma membrane proteins composed of six predicted transmembrane domains, a C-terminal FAD binding domain and two N-terminal calcium-binding (EF-hand) domains. Among the ten members of the RBOH gene-family in Arabidopsis (i.e. RBOHA– RBOHJ), RBOHD and RBOHF were found to play a crucial role in the generation of apoplastic ROS

triggered by *avirulent* strains of *Pseudomonas syringae* and *Hyaloperonospora arabidopsidis* (Torres et al., 2005; Pogány et al., 2009; Chaouch et al., 2012; Marino et al., 2012). Plants can detect foreign pathogens via the recognition of PAMPs by surface-localized receptor-like kinases (RLKs), which comprise of a ligand-binding ectodomain and an intracellular kinase domain. The Arabidopsis leucine-rich repeats (LRR)-RLK FLS2 (FLAGELLIN-SENSITIVE2) ectodomain can recognize and directly bind to flg22, a conserved 22-amino acid epitope from bacterial flagellin. In Arabidopsis mutants lacking a functional RBOHD, the flg22-induced ROS burst is completely blocked. A recent study revealed that the receptor-like cytoplasmic kinase, BIK1 (BOTRYTIS-INDUCED KINASE1), a component of the FLS2 immune receptor complex, mediates phosphorylation of RBOHD in a calcium-independent manner to enhance ROS generation; and such site-specific phosphorylation of RBOHD also regulated Ca^{2+} influx and contributed to BIK1-regulated stomatal closure (Kadota et al., 2014; Li et al., 2014). Likewise, genetic analyses suggested that RBOHD and RBOHF were involved in regulation of stomatal closure induced by ABA, which has been shown to induce production of H_2O_2 in guard cells (Murata et al., 2001; Yanyan Zhang et al., 2009). ABA-induced stomatal closure was impaired in the *rbohf* and even stronger in *rbohd rbohf* double mutant (Kwak et al., 2003); furthermore application of the NADPH oxidase inhibitor diphenyliodonium (DPI) produced similar effect on ABA-induced stomatal closure (Zhang et al., 2001). RBOHD was also identified as a major component in mediating a systemic ROS signaling in plants (Miller et al., 2009). Similar apoplastic ROS signaling can be activated by exposure to a gaseous ROS molecule O_3, which enter through stomatal pore and rapidly degrades into $O_2^{\bullet-}$ and H_2O_2 in the apoplast (Wohlgemuth et al., 2002). The resultant apoplastic ROS signals (i.e. H_2O_2) can translocate inside the cells through water channel or activation of different subunits of heterotrimeric G proteins (Joo et al., 2005; Dynowski et al., 2008). ROS production is an early signal event in the apoplast shared among different biotic and abiotic stresses. To study the role of apoplastic ROS-mediated defense signaling, both flg22 and O_3 can be applied as tools to initiate ROS signaling (Sierla et al., 2013; Vainonen and Kangasjärvi, 2014).

Cell wall peroxidases regulate another source of the production of apoplastic ROS production. Transgenic Arabidopsis plants expressing an anti-sense cDNA targeting type III peroxidases exhibited a diminished oxidative burst and enhanced susceptibility to flg22 and *Fusarium oxysporum* compared with Landsberg erecta (wild-type) (Bindschedler et al., 2006). Further studies of the antisense line revealed decreased expression of PEROXIDASE33 (PRX33) and PRX34. Indeed, the expression of *PRX33* and *PRX34*, as well as *RBOHD* was significantly induced by *F. oxysporum* elicitor within two hours in wild-type tissue culture cells. (O'Brien et al., 2012). Wild-type, *prx33* and *prx34* culture cells treated with sodium azide (a peroxidase inhibitor) exhibited lower production of H_2O_2 in comparison to DPI treatment in response to *F. oxysporum* elicitor. Likewise their basal levels of H_2O_2 were lower than the WT in culture cells of both mutants (O'Brien et al., 2012). In addition, compared to *rbohD* and *rbohF* T-DNA mutant, the *prx33* and *prx34* T-DNA mutants were more susceptible to *Pseudomonas syringae* infection and exhibited reduced MAMP-elicited transcription of defense-related genes and callose deposition, as it could be restored by exogenous H_2O_2 application (Chaouch et al., 2012; Daudi et al., 2012). Overall, this suggests different roles for RBOHs and peroxidases in the regulation of apoplastic ROS production induced by different pathogens.

1.1.2 Ion fluxes and ion channels in response to apoplastic ROS

In addition to PM-bounded RLKs, rapid ion fluxes play key roles in the initiation of stress signal transduction cascades and hormone-signaling (Lüthje et al., 2013). Pathogen entry into host tissue through stomata is a critical first step in causing infection in plants (Melotto et al., 2006). Stomatal closure is one of the most-efficient defense mechanisms in the response to harmful stimuli. Guard-cell ion-channels are a good example of typical ion signaling activated in response to apoplastic ROS signal elicited by flg22 or O_3 (Song et al., 2014). Abscisic acid (ABA) plays important roles in regulation of stomatal closure, consequently much attention has been given to ABA signaling associated with the regulation of ion channels in the guard cell. For example, after application of ABA or O_3 rapid stomatal closure can be induced within 10 min (Vahisalu et al., 2008; Kollist et al., 2014). This rapid process include the production of apoplastic ROS, activation of S- and R-type ion channels (Vahisalu et al., 2008), triggering K^+ efflux (Schwartz et al., 1994) and increased $[Ca^{2+}]_{cyt}$ stimulated by Ca^{2+} permeable channels. (Pei et al., 2000; Kwak et al., 2003). In the absence of ABA, type 2C protein phosphates (PP2Cs) including ABI1 (ABA INSENSITIVE 1) and ABI2, keep the ABA signaling pathway turned off through inactivation of SnRK2 (SNF-related kinases) including OST1 (OPEN STOMATA 1) (Murata et al., 2001; Vahisalu et al., 2008; Vahisalu et al., 2010). Binding of ABA by PYR/PYL/RCAR (PYRABACTIN RESISTANCE/PYR1 LIKE/REGULATORY COMPONENT OF ABA RECEPTOR) stimulates formation of a complex between these receptors and PP2C phosphatases. This leads to inactivation of the PP2Cs, and activation of OST1, which acts as a positive regulator of stomatal closure (Kollist et al., 2014).

NADPH-dependent ROS production is essential for the regulation of stomatal closure, i.e. ABA-induced stomatal closure was impaired in both *rbohd* and *rbohd rbohf* double mutants, while function could be restored by the application of exogenous H_2O_2 (Kwak et al., 2003). The accumulation of $[Ca^{2+}]_{cyt}$ was also diminished in both *abi1, abi2* and *robhd*/*rbohf* mutant in response to ABA (Murata et al., 2001; Kwak et al., 2003). Further studies revealed that OST1 could phosphorylate multiple amino acids in the N terminus of the S-type anion channel SLOW ANION CHANNEL-ASSOCIATED 1 (SLAC1) (Vahisalu et al., 2010). However, SLAC1 is not only activated by OST1, but also activated by elevated $[Ca^{2+}]_{cyt}$ and Ca^{2+}-dependent protein kinases (CDPKs)(Geiger et al., 2010). Similar to ROS, altered $[Ca^{2+}]_{cyt}$ is another critical step towards initiating defense signaling induced by a specific stimulus or environmental cues. Ca^{2+} influx appears to be controlled by PM-localized glutamate receptor-like proteins (GLRs), cyclic nucleotide gated-ion channels (CNGCs) and vacuolar TWO-PORE CHANNEL 1 (TPC1)(Steinhorst and Kudla, 2013; Choi et al., 2014). So far, very limited evidence has been obtained for the role of GLRs and TPC1 in plant immunity (Kong et al., 2015). This suggests a role for the members of the CNGCs family as the strongest candidates for regulation of inward Ca^{2+} flux in plant defense responses. Among the 20 members in this family, CNGC2 was the first Ca^{2+} channel functionally characterized with three different heterologous expression systems (Leng et al., 1999). Arabidopsis *defense no death1* (*dnd1*) a null mutant in the *CNGC2*/*DND1* gene; has impaired cyclic nucleotide monophosphate-dependent Ca^{2+} influx and reduced Ca^{2+} accumulation in leaves (Yu et al., 1998; Ali et al., 2007; Ma et al., 2010). In addition, the *dnd1* mutant has constitutively activated expression of pathogenesis-related (*PR*) genes and an elevated SA content (Yu et al., 1998). Likewise the null mutation in *CNGC4*/*DND2* confers impaired Ca^{2+} signaling and constitutive defense responses (Chin et al., 2013). Interestingly, in addition to impaired inward Ca^{2+} flux in *dnd1*, both flg22-induced ROS production and O_3-triggered

apoplastic ROS signaling were abolished in this mutant (Mersmann et al., 2010; Wrzaczek et al., 2010), suggesting an important role for the CNGC2 ion channel in apoplastic ROS signaling.

Overall, this raises the question: how can a simple ion like Ca^{2+} regulate multiple signaling pathways as part of a defense response? One idea is that a toolkit of different Ca^{2+}-binding proteins function as Ca^{2+} sensors and bind Ca^{2+} via a helix-loop-helix EF-hand in order to initiate specific responses. This involves the CDPK gene family, calmodulin proteins (CaMs) - calmodulin-like proteins (CMLs) and calcineurin B-like proteins/CBL-interacting protein kinases (CBL/CIPKs) complexes (Steinhorst and Kudla, 2013). These Ca^{2+} sensors could activate RBOHs and thus facilitate ROS production in innate-immunity signaling. For example, CDPK5 can regulate Ca^{2+}-dependent phosphorylation of RBOHD (in Arabidopsis) and RBOHB (in *Nicotiana benthamiana*) to activate ROS production induced by flg22 and H_2O_2 (Kobayashi et al., 2007; Dubiella et al., 2013). Furthermore, RBOHF can be activated via Ca^{2+}-binding and phosphorylation by CBL/CIPK complexes (Drerup et al., 2013). Reciprocally, when apoplastic ROS production is eliminated via DPI application or mutation in RBOHD, this led to the loss of a second Ca^{2+} peak induced by flg22, demonstrating a feedback effect of ROS on Ca^{2+} signaling. However, this feedback-loop regulation is not simply the consequence of a ROS-induced increase in $[Ca^{2+}]_{cyt}$ per se, rather it is modulated through spatiotemporal mechanism (Short et al., 2012). For example, gene expression analysis revealed that O_3, H_2O_2, and cold could trigger different Ca^{2+} signatures, which may serve as intermediates to transduce different stress-induced signals to the transcription machinery and initiate corresponding defense activation. The magnitude and temporal dynamics of stress-induced ROS and Ca^{2+} signaling provides a flexible system for plant to cope with external stimuli and environmental cues. Overall these data suggest a central role for interaction between cytosolic Ca^{2+} signaling and apoplastic ROS production in the regulation of stomatal movement and initiation of defense responses.

1.1.3 Intracellular ROS homeostasis and activation of signaling cascades in response to apoplastic ROS

Stress-induced formation of ROS in the cytosol may trigger a redox imbalance, resulting in trancriptome-reprogramming and/or PCD (Vaahtera et al., 2014; Vainonen and Kangasjärvi, 2014). In addition to the stress-induced ROS burst, plants produce a large amount of ROS as a result of photosynthesis and metabolism. Sources of intracellular ROS include $^1O_2$ generated from photodynamic excitation of O_2 in photosystem II during photosynthesis; $O_2^{\bullet-}$ generated at photosystem I and II of chloroplast; $O_2^{\bullet-}$ generated at complexes I and III of the mitochondrial electron transport chain (ETC); and $O_2^{\bullet-}$ generated in reaction that is catalyzed by xanthine oxidase in peroxisome. $O_2^{\bullet-}$ is rapidly converted to H_2O_2 and O_2 by SOD (Vaahtera et al., 2014; Demidchik, 2015). Accordingly, plant cells need ROS scavenging system(s) to handle the high rate of ROS generation that already occurs in non-stressed plants. This scavenging system consist of both enzymatic and nonenzymatic antioxidants; including SODs, catalases and low-molecular-weight molecules such as ascorbic acid (vitamin C), α -tocopherol, glutathione, carotenoids and phenolic compounds (Ahmad et al., 2010). Compared to the other ROS, H_2O_2 is a more stable non-radical molecule and its half-life is controlled by the activities of catalases (CAT) and peroxidases (APX) (Rahman et al., 2005; Demidchik, 2015). Plants deficient in catalases exhibit elevated levels of H_2O_2 and intracellular redox perturbation, that can be triggered by switching from high CO_2 conditions (which inhibit photorespiration) to ambient air

or from different light fluence rates (Queval et al., 2007). Interestingly, double antisense plants lacking both APX and CAT exhibited less susceptibility to oxidative stress than single antisense APX or CAT plants, which is coupled with inhibition of photosynthetic metabolism (Rizhsky et al., 2002). In contrast, double mutant lacking thylakoid ascorbate peroxidase (*tylapx*) and cytosolic ascorbate peroxidase1 (*apx1*) results in enhanced sensitivity to oxidative stress and retarded growth (Miller et al., 2007). These data indicate that the cytosolic H_2O_2 originating from the peroxisomes or chloroplasts, could function as a redox messenger among different organelles.

Another facet of ROS regulation in the cytosol is provided by activation of mitogen-activated protein kinases MAPKKK-MAPKK-MAPK signaling cascades that link upstream receptors and downstream targets. Genetic analysis has revealed that several predominant MPKs are shared between biotic- and abiotic-stresses responses, including the MAPKKK MEKK1; the MAPKKs MKK1, 2, 4, and 5; and the MAPKs MPK3, 4, and 6. For example, MEKK1-MPK4 kinase activity is activated by flg22 and H_2O_2 (Asai et al., 2002; Nakagami et al., 2006); and *mekk1* plants exhibit increased accumulation of H_2O_2 and their ROS-induced MAPK MPK4 activation is compromised (Nakagami et al., 2006). Likewise, the *mpk4* mutant has similar dwarfism, PCD-associated accumulation of SA and H_2O_2 as the *mekk1* plants, suggesting that MEKK1 functions upstream of MPK4 and downstream of ROS signals. In addition, O_3 treatment triggers the rapid activation of MPK3 and MPK6 within two hours and induces translocation of these kinases from cytosol to nucleus (Ahlfors et al., 2004). However, flg22-induced activation of MPK3 and MPK6 is not affected in *rbohd* (Xu et al., 2014), suggesting that RBOHD-independent signaling pathways could be involved in the activation of MPK3 and MPK6. In contrast, another MAPK, the mechanical-wound-activated MPK8, negatively regulates ROS accumulation via RBOHD, and its full activation requires direct binding of CaM and MKK3 (Takahashi et al., 2011). An even more complex role of MAPKs is evident in its synergistic or antagonistic interaction with different hormones and the regulation of hormone synthesis. For example, the stability of the important ethylene biosynthesis enzymes ACS2 and ACS6 (ACC synthase), are regulated by MPK3 and MPK6 through direct phosphorylation (Han et al., 2010). However, another study revealed that a MKK9–MPK3/MPK6 cascade promotes EIN3 (ethylene insensitive 3) mediated transcription of ethylene signaling (Yoo et al., 2008). In addition, the activity of MEKK1-MPK4 is not only required for the accumulation of SA, but also needed for regulation of the JA and ethylene responses (Brodersen et al., 2006; Gawroński et al., 2014). These data provide links to MAPK-signaling cascades, ethylene/SA/JA biosynthesis and signaling and intracellular ROS.

1.2 Integration of ROS with hormonal signaling

The complex interface between ROS, redox and hormone-signaling pathways strongly influence the outcome of stress responses, including establishment of effective defenses or activation of PCD. Modulation of hormone homeostasis appears to be one of the dominant features in the regulation of defense response, which is used by the plant to prioritize and balance its energy flow in order to optimize growth and defenses. Mounting evidence reveals the roles of SA, JA and ethylene in stress-induced PCD and transcriptome reprograming (Wang et al., 2006). However, an integrated view on the spatiotemporal dynamics of hormone production and signaling during the early defense response is still lacking, especially in relation to how these hormones interact with early apoplastic ROS signals.

1.2.1 The role of SA in response to apoplastic ROS

1.2.1.1 SA biosynthesis and metabolism

SA is a phenolic acid and functions as a crucial signaling molecule with multiple roles in activation of the defense, hypersensitive response (HR, in which necrotic lesions form at the sites of pathogen entry), plant growth and development, photosynthesis and respiration, regulation of ion channels (Rivas-San Vicente and Plasencia, 2011). There is substantial variation in SA content among different species and different mutants within same species (Raskin et al., 1990; Rivas-San Vicente and Plasencia, 2011). Intriguingly such natural variation for response to SA is also detected among Arabidopsis accessions, which could be associated with plant-pathogen interaction related to geographical population structures (van Leeuwen et al., 2007; Narusaka et al., 2013).

SA can be synthesized through two distinct pathways that employ different precursors catalyzed from chorismate, which is the terminal metabolite of the shikimate pathway (Tzin and Galili, 2010; D'Maris Amick Dempsey et al., 2011). The phenylalanine ammonia-lyase (PAL) pathway is the first pathway identified in SA synthesis (Pellegrini et al., 1994; D'Maris Amick Dempsey et al., 2011). Simultaneous mutation of all four Arabidopsis *PAL* (phenylalanine ammonia-lyase) genes (*pal1 pal2 pal3 pal4*) results in substantially reduced but not complete elimination of SA after infiltration of avirulent *Pst DC3000 avrRpt2* (Huang et al., 2010). The second SA biosynthesis pathway, the isochorismate (IC) pathway, is generally believed to be the primary route for the formation of SA in Arabidopsis. Its key regulatory enzymes are two chloroplast-localized enzymes ICS1 (isochorismate synthase) and ICS2 (Wildermuth et al., 2001; Garcion et al., 2008). The accumulation of SA (<90%) is severely impaired in *ics1*/*sid2* and *ics1 ics2* in response to UVB and avirulent strains of *Pseudomonas syringae* (Garcion et al., 2008). Apart from SA synthesis, ICS1 and ICS2 are also involved in phylloquinone production (another isochorismate-derived end product), which functions as an electron acceptor and forms an essential part of photosystem I (Garcion et al., 2008). The growth retardation and lack of phylloquinone in *ics1 ics2* double mutant indicate that SA synthesis may play an important role, either directly or indirectly, in maintaining equilibrium between defense and growth.

1.2.1.2 The role of SA in defense responses and ROS signaling

The role of SA in defense responses is first revealed following application of SA or its derivative acetyl-salicylic acid (aspirin) to *Tobacco cv. Xanthi-nc* which dramatically increases its resistance to tobacco mosaic virus (TMV)(White, 1979). Later studies suggest that removing SA through expression of the bacterial SA-degrading enzyme salicylate hydroxylase (*NahG*) in Arabidopsis and tobacco compromised the resistance to viral, fungal, and bacterial pathogens (Seskar et al., 1998). Furthermore, this makes the plants unable to induce systemic acquired resistance (SAR), a mechanism that confers protection to uninfected parts of the plant (Delaney et al., 1994; Yang et al., 1997). To address how SA activates disease resistance, an enormous number of studies have been carried out to dissect the essential components in SA-mediated signaling pathway in relation to plant-pathogen interactions (Vlot et al., 2009). Accumulation of ROS and SA is often associated with killing invading pathogen and/or activating cell wall lignification of infection

sites during SAR and HR (Brisson et al., 1994; Durner et al., 1997; Shirasu et al., 1997). However, the relationship between SA and ROS is complicated. It was proposed that high levels of SA could induce H_2O_2 accumulation through binding and inhibition of the H_2O_2 scavenging enzyme catalase (Chen et al., 1993; Yansha Li et al., 2013). On the contrary, other studies suggested that physiological levels of SA may not be sufficient to directly suppress catalase activity (Summermatter et al., 1995; Rao et al., 1997). Intriguingly, lesion formation due to the accumulation of H_2O_2 in the catalase deficient mutant *cat2* can be reduced by the introduction of *ics1*/*sid2*, which indicates that during stressed conditions, the self-amplification loop between ROS and SA could be regulated in a redox-dependent manner (Chaouch et al., 2010). Thus, catalases could therefore function as a general target of SA instead of specific SA receptors in plants. To identify key regulators of SA-mediated signaling, forward genetics screens were employed to find positive regulator(s) of SA-regulated PR genes. This led to the isolation of *npr1* (nonexpresser of PR genes) and the allelic mutant *nim1* (Cao et al., 1994; Delaney et al., 1995). The insensitivity of *npr1* to various SAR-inducing treatments and increased susceptibility to pathogen infection indicated that NPR1 is the master regulator of SAR (Cao et al., 1997). However, a recent study found no considerable SA-binding activity for NPR1 using a ligand-binding assay (Fu et al., 2012). Instead of direct binding, SA has been shown to regulate translocation of NPR1 between the cytoplasm and the nucleus through cellular redox changes (Spoel and Dong, 2012). Intriguingly, a separate study reported that NPR1 could bind SA in an equilibrium dialysis assay (Yue Wu et al., 2012). Therefore, whether NPR1 functions as a direct SA receptor still remains elusive. The redox changes induced by SA allow NPR1 to switch reversibly from an oligomeric complex to a monomeric state in the cytoplasm (Mou et al., 2003). The monomeric NPR1 is translocated to the nucleus to form a complex with TGA transcription factors (TFs), which regulate further transcriptome reprograming and defense responses. Recently, NPR1 homologs NPR3 and NPR4 are reported to function as SA receptors with low and high SA affinities respectively, which in turn regulate NPR1 protein degradation (Fu et al., 2012). Accordingly this model provides evidence that the balance between the abundance of NPR1, its oligomer to monomer transition and different levels of SA could help the plant switch between growth and defense under different type(s) and/or intensities of stresses.

Some of the regulatory components upstream of SA-mediated signaling are initially identified through genetic screens searching for mutants with altered pathogen resistance. For example, ENHANCED DISEASE SUSCEPTIBILITY1 (EDS1) and its interacting partner, PHYTOALEXIN DEFICIENT4 (PAD4), constitute a regulator hub that is required for disease resistance (R) gene-mediated disease resistance (Glazebrook et al., 1996; Falk et al., 1999; Wiermer et al., 2005). The multiple phenotypes of *eds1* and *pad4* indicate a regulatory role for EDS1/PAD4 in pathogen-induced SA production, disease resistance and defense signaling (Parker et al., 1996; Zhou et al., 1998; Feys et al., 2001; Rietz et al., 2011). These regulators are also known to coordinate chloroplast-associated ROS homeostasis and H_2O_2-aossicated cell death (Mühlenbock et al., 2008). The run-away cell death phenotype of the *lesion simulating disease1* (*lsd1*) mutant is modified by *eds1* or *pad4*, which points to EDS1/PAD4 being positive regulators of ROS-triggered cell death (Rustérucci et al., 2001). EDS1 executes its function both in the cytosol and nucleus, thus accurate defense response requires accurate coordination of several cellular compartments (Heidrich et al., 2011).

Other regulators of SA accumulation and SA signaling are identified through screens for mutants with constitutive defense responses, for example through identification of mutants with high

expression of the SA-inducible *PR1* or *PR2* genes. These include the *cpr* (*constitutive expressor of PR genes*) and *cim* (*constitutive immunity*) mutants (Bowling et al., 1994; Maleck et al., 2002). The dwarf phenotype and high SA content of *cpr1*, *cpr5* and *cpr6* is less pronounced when these plants are grown under high light, whereas mutants with low levels of SA (*NahG* and *sid2-2*) were impaired in acclimation of high light (Mateo et al., 2006). Thus, light and photo-oxidative stress interacts with SA signaling. Moreover, plants that overexpress *NUDT7* (*NUDIX HYDROLASE HOMOLOG 7*) are protected from damage caused by treatment with paraquat that causes ROS to form in the chloroplast (Ishikawa et al., 2009). Conversely, the *nudt7* mutant is sensitive to paraquat treatment. Introduction of *eds1* into *nudt7* alleviates chloroplast derived $O_2^{\bullet-}$ and H_2O_2 accumulation, dwarfism and RBOHD dependent-cell death in this mutant (Straus et al., 2010). Collectively this pattern shows that EDS1 is a regulator of defense and cell death responses in a variety of contexts.

Another disease-resistant mutant, *aberrant growth and death2* (*agd2-1*) has elevated SA content, altered leaf morphology and mild dwarfism. The close homolog ALD1 (AGD2-LIKE DEFENSE RESPONSE PROTEIN 1) encodes an aminotransferase; and is partially responsible for the elevated SA content and a majority of the disease resistance and dwarfism of *agd2*-1 (Song et al., 2004). In addition, the *ald1* mutant has impaired accumulation of SA in distal leaf tissue after infection with *P. syringae*, which can be restored by the exogenously application of pipecolic acid (Pip) prior to the pathogen treatment (Návarová et al., 2012). The cyclic non-protein amino acid L-Pip is an ALD1-dependent bioactive product, which is the only amino acid found to substantially increase in leaves distal from sites of pathogen inoculation. Concomitant with SAR, application of Pip alone also triggers accumulation of SA and camalexin and induced expression of PR genes. Like ALD1, plants lacking flavin-dependent monooxygenase (FMO1) fail to induce Pip-triggered systemic accumulation of SA and systemic expression of diverse defense-related genes (Mishina and Zeier, 2006), which cannot be rescued with application of Pip, suggesting that FMO1 functions downstream of ALD1. Importantly, ALD1 overexpressing plants exhibit increased disease resistance and pronounced ROS production without producing additional Pip. In contrast, the *ald1* mutant exhibits reduced production of ROS induced by flg22 compare to wild type (Cecchini et al., 2014). Thus it is probable that in early defense responses, there is a positive feedback-amplification-loop involving SA, ROS and Pip as the central players.

1.2.1.3 Role of SA and apoplastic ROS in triggering cell death

PCD and the pathogen associated HR, both genetically regulated cellular suicide, is often found to be associated with an accumulation of ROS and SA. Substantial effort has been made to uncover the signaling components involved in regulation and execution of cell death directly in contact with, or close to the pathogen (Dickman and Fluhr, 2013). Lesion mimic mutants (LMMs), mutants that display spontaneous development of lesions, provide valuable genetic tools to dissect various aspects of PCD and pathogen resistance pathways. All LMMs exhibit similarly constitutive activation of defense and spontaneous cell death that resembles HR after pathogen infection (Bruggeman et al., 2015). However, the pathways that activate cell suicide are versatile, such as involvement of the chloroplast and energy transduction, impaired signal perception at PM, and disruption of biosynthesis of fatty acids or Ca^{2+} signaling (Bruggeman et al., 2015). Furthermore, isolation of suppressors of LMM phenotypes has unraveled highly complex networks that regulate PCD. Removal of SA in many LMMs through expressing *NahG* or

introduction of *sid2*, can alleviate the death and dwarfism phenotypes, suggesting SA signaling functions as central hub in PCD execution (Bruggeman et al., 2015).

Exposure to acute concentrations of O_3 causes lesion formation in sensitive plants, which show similarities to the HR lesions produced in plant-pathogen interaction (Overmyer et al., 2005). Entering through stomata, O_3 is rapidly degraded into secondary ROS and leads to an apolastic ROS burst in the guard cells and mesophyll cells. Early studies have shown that O_3-induced cell death in the sensitive Arabidopsis accession Cvi-0 occurs through a SA-dependent pathway; the cell death is proportional to the accumulation of SA and ROS (Rao and Davis, 1999). Intriguingly removal of SA in O_3-tolerant and O_3-sensitive accessions had contrasting effect on the lesion formation induced by O_3 (Rao et al., 2000). For example, expressing *NahG* in Cvi-0 relieved its O_3-hypersensitivity, while Col-0:*NahG* was sensitive to O_3 compare to Col-0, suggesting that SA plays a dual role in O_3 responses. Elevated levels of SA appear to promote cell death, but basal level of SA is required to activate defense responses. However, the O_3-triggered transcriptional responses and cell death are completely blocked in the LMM *dnd1* (Overmyer et al., 2005; Wrzaczek et al., 2010), indicating the role of SA in modulating cell death is not simply dose-dependent; instead it could determine the balance between life or death depending on when and how the stress was initiated. Furthermore, flg22-induced ROS production was impaired in *dnd1*, suggesting that ROS production and SA could activate separate signaling pathways that exhibit negative crosstalk in specific conditions. Many studies focus on cell death at late time points after onset of the treatment, but further studies are required to address the role of SA and/or other signaling components during the early defense response.

1.2.2 Role of JA in response to apoplastic ROS

1.2.2.1 Biosynthesis of JA and signaling

JA and its biologically active form, a conjugate with isoleucine (JA-Ile) are oxylipins derived from lipid oxidation. They are involved in cell growth and stress responses, including root growth inhibition, trichome initiation, anther development, wounding response, and regulation of cell death and plant-pathogen interactions. The biosynthetic pathway of JA/JA-Ile includes the following key steps: the first half of JA biosynthesis takes place in plastids, initiated from α-linolenic acid (18:3), which is catalyzed by plastid-located lipoxygenases (LOXs); the product is processed by ALLENE OXIDE SYNTHASE (AOS) and ALLENE OXIDE CYCLASE (AOC) to form cyclopentenone cis-(+)-12-oxophytodienoic acid (OPDA); OPDA is further catalyzed by peroxisome-localized OPDA reductase3 (OPR3). In the last step JASMONATE RESISTANT 1 (JAR1) forms the conjugate (+)–7-*iso*-JA-Ile. The perception of JA-Ile by the SCF^{COI1}–JAZ (JA ZIM domain) co-receptor complex leads to JA/JA-Ile-induced gene expression (Wasternack and Hause, 2013). Several key components of this functional co-receptor complex have been characterized, such as the JA-Ile receptor COI1 (CORONATINE INSENSITIVE 1), JAZ1 and MYC2/Jasmonic insensitive 1 (JIN1). The positive regulator of JA signaling, MYC2/JIN1 (a basic helix-loop-helix (bHLH) transcription factor), is repressed by JAZ1, upon perception of stress-induced JA-Ile production by COI1, JAZ1 is degraded through the proteasome and MYC2 is released to activate gene expression (Wasternack and Hause, 2013).

Mutations in several of these key components cause impaired regulation of defense responses and development. For example, mutants deficient in JA biosynthesis such as *aos*/*dde2* or *opr3* are male sterile that can be restored by JA treatment during floral development (Xie et al., 1998). MYC2 was first identified in a mutant screen for reduced sensitivity of JA-induced root-growth inhibition (Berger et al., 1996). So far MYC2 has been considered to be the master switch in JA signaling due to its important role in defense response against herbivores, pathogens and in linking JA signaling to other signaling pathways (Dombrecht et al., 2007; Chen et al., 2011; Kazan and Manners, 2012). MYC2 probably acts redundantly with the homologous proteins MYC3 and MYC4, since the *myc2 myc3 myc4* triple mutant is more strongly impaired in JA signaling than the corresponding single mutants (Fernández-Calvo et al., 2011). In addition to MYC2, there is a parallel signaling pathway conferred by the TFs OCTADECANOID-RESPONSIVE ARABIDOPSIS59 (ORA59) and ETHYLENE RESPONSIVE FACTOR1 (ERF1). Both TFs constitute an important regulatory hub for a JA-ethylene-induced defense program. ORA59 and ERF1 can bind to the GCC box in the promoter region of JA-responsive marker gene PLANT DEFENSIN 1.2 (PDF1.2), which is highly sensitive to suppression by SA (Spoel et al., 2003). Furthermore, the MKK3-MPK6 cascade is also involved in regulation of JA biosynthesis, and occurs in the JA-dependent negative regulation of MYC2 (Takahashi et al., 2007).

1.2.2.2 The role of JA in regulation of cell death and apoplastic ROS signaling

Lipid oxidation in membranes caused by ROS or wounding, rapidly activates JA-mediated pathways (Glauser et al., 2008; Farmer and Mueller, 2013). The precise mechanism of JA in the regulation of cell death is not yet completely clear due to conflicting reports of its effect on regulation of cell death. Acute O_3 exposure induces rapid expression of *LOXs* and production of JA within 3 hours (Rao et al., 2000), which could be associated to suppression of O_3 induced cell death. The possible role of JA in ROS-induced cell death is analyzed using mutant defective in JA biosynthesis or perception and by exogenous application of MeJA. Pretreatment of the O_3-senstivie Cvi-0 with 200µM MeJA can attenuate O_3-induced cell death as well as accumulation of H_2O_2 and SA, whereas JA suppressed cell death did not occur in plants expressing *NahG* (Rao et al., 2000). The inhibition of PCD by JA could therefore be partially achieved through inhibition of the ROS-SA self-amplification loop. The protective role of JA during oxidative stress was further tested by exposing mutants defective in JA signaling to O_3 and the superoxide generator methyl viologen (Paraquat). The JA insensitive *coi1* and jasmonic acid-biosynthesis-defective *jar1*, *opr3* and *fad3 fad7 fad8* mutants are all highly sensitive to acute O_3 (Rao et al., 2000; Overmyer et al., 2005). Similarly the involvement of JA is supported by studies where pretreatment with MeJA conferred paraquat tolerance to Arabidopsis. Another hypothesis for involvement of JA in the protection against O_3 and paraquat-induced cell death suggests the coordinated activation of production of antioxidants (Sasaki – Sekimoto et al., 2005). In contrast to the JA-induced suppression of O_3 and $O_2^{\bullet-}$/H_2O_2-dependent cell death, the possible role of JA as promoter of cell death is investigated by crossing *flu* with *aos* mutant. The *fluorescent* (*flu*) mutant has enhanced production of $^1O_2$ in photosynthetic tissues and spontaneous cell death when growth conditions are switched from dark to light (Meskauskiene et al., 2001). The *flu aos* double mutant exhibits less $^1O_2$ mediated cell death (Danon et al., 2005). Another example of increased cell death by JA is found in the rice LMM *cea62* (*constitutive expression of aos gene62*) due to over accumulation of JA (Liu et al., 2012). Thus, JA may either have a role protecting against or promoting cell death, dependent on treatment or mutant background.

1.2.3 The role of ethylene in response to apoplastic ROS

1.2.3.1 Ethylene biosynthesis and signaling

The gaseous hormone ethylene is another essential regulator involved in stress responses and development. Enhanced ethylene biosynthesis is often observed in stress-challenged plants. Ethylene biosynthesis is initiated from the amino acid methionine, which is converted to ethylene through a two-step biochemical pathway involving conversion of S-adenosyl-L-methionine (SAM) to 1- aminocyclopropane-1-carboxylic acid (ACC) and subsequent oxidative cleavage of ACC to form ethylene. The enzymes catalyzing these two reactions are ACC synthase (ACS) and ACC oxidase (ACO), respectively (Broekaert et al., 2006). ACS, encoded by a group of genes, is the first dedicated step and generally considered as the rate-limiting step in ethylene biosynthesis (Chae and Kieber, 2005). The activity of this enzyme is associated with stress-triggered ethylene production. For example, ACS2 and ACS6, were previously shown to be phosphorylated and stabilized by MPK3 and MPK6 (Liu and Zhang, 2004). Further studies revealed that WRKY33 was involved in expression of *ACS2* and *ACS6* through direct binding to the W-box in the *ACS2* and *ACS6* promoter region (Li et al., 2012). In Arabidopsis, ethylene is perceived by five ER membrane or Golgi apparatus-localized receptors: ETHYLENE RESPONSE1 (ETR1), ETHYLENE RESPONSE SENSOR1 (ERS1), ETR2, ERS2, and ETYLENE INSENSITIVE4 (EIN4) (Cho and Yoo, 2014). In the absence of ethylene, these receptors act redundantly to negatively regulate the signaling pathway by activating CONSTITUTIVE TRIPLE RESPONSE1 (CTR1). Upon binding ethylene, the negative function of the receptor-CTR1 complex is inactivated, leading to cleavage of the membrane protein ETHYLENE-INSENSITIVE2 (EIN2) releasing the C-terminus (EIN2C) to translocate to the nucleus to regulate ethylene gene expression (Qiao et al., 2009; Qiao et al., 2012). A null mutation of CTR1 leads to constitutive cleavage and nuclear localization of EIN2C, leading to EIN3 and EIN3-LIKE1-dependental activation of ethylene responses (Qiao et al., 2012). The elimination of ethylene sensitivity in *ein2* suggests an essential role of this protein as a positive regulator of ethylene responses (Alonso et al., 1999).

1.2.3.2 The role of ethylene in regulation of cell death and apoplastic ROS signaling

Another type of PCD is senescence that partially shares similar physiological events with HR. Early studies reported that ethylene production was associated with the initiation and progression of leaf senescence in plants (Aharoni and Lieberman, 1979; Koyama, 2014). In cell death, the Raf-like MAPKKK ENHANCED DISEASE RESISTANCE 1 (EDR1) encodes a CTR1-like kinase that functions as a negative regulator of plant defense. The *edr1* mutant displays ethylene-induced spontaneous cell death, which can be suppressed by *ein2* (Tang et al., 2005). EDR1 also negatively affects MKK4/MKK5 protein levels. A recent study further showed that MKK4/MKK5 physically associated with EDR1 and negatively regulated the MAPK cascade to modulate resistance and mildew-induced cell death (Tang et al., 2005; Zhao et al., 2014). Likewise, another LMM *sr1* (SIGNAL RESPONSIVE1, also known as CALMODULIN BINDING TRANSCRIPTION ACTIVATOR3 [CAMTA3]) regulates ethylene-induced senescence by directly binding to the EIN3 promoter region in vivo. The enhanced senescence of *sr1* can be reduced by introduction of *ein3* (Nie et al., 2012). Intriguingly hyper-accumulation of ethylene in the

ethylene overproducer (*eto1*) and *eto3* mutants upon acute O_3 exposure is proportional to SA level in these mutants. By contrast, Col-0:*NahG* and *npr1* are impaired in ethylene accumulation in response to acute O_3 treatment (Rao et al., 2002), suggesting that SA-mediated signaling is required for ethylene accumulation during stress. In addition, blocking ethylene perception in the O_3-sensitive mutant *jar1* can prevent the spread of cell death (Tuominen et al., 2004). These data suggest that ethylene acts in concert with several signaling and modulating plant immune responses.

Overall, the plant hormones SA, JA, and ethylene have pivotal roles in the regulation of apoplastic ROS signaling. However, hormone-mediated signaling pathways are interconnected in a complex network (summarized in Figure 1). This provides plants with an enormous regulatory potential to rapidly respond to environmental cues. This synergism and antagonism among the three hormones has prevented the precise quantification of the effects of the three hormones on apoplastic ROS signaling. Hence, a precise delivery system for apoplastic ROS would allow the role of ROS to be examined without confounding effects from concurrent activation of other signaling pathways.

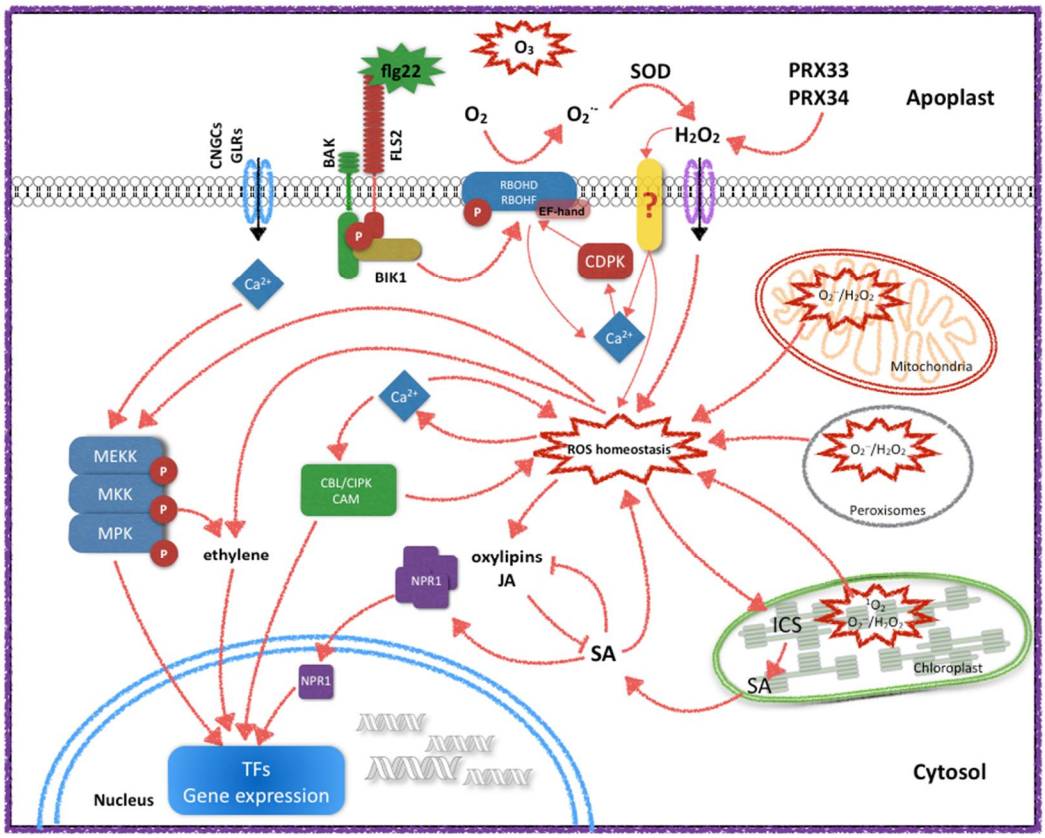

Figure 1. Summary of the apoplastic ROS-triggered signaling pathways. The apoplastic ROS burst can be activated by exposure to O_3, which enters leaf through stomatal pores and rapidly degrades into $O_2^{\bullet-}$ and H_2O_2. Likewise, Flg22 perception triggers phosphorylation of the cytoplasmic domains of FLS2, BAK1, and BIK1, leading to phosphorylation and activation of the PM-localized NADPH oxidases (RBOHD). Subsequently the activated RBOHs transfer electrons from cytoplasmic NADPH to apoplastic O_2, generating $O_2^{\bullet-}$ in the apoplastic side of PM, which is dismutated into H_2O_2 by SOD.

In addition, apoplastic peroxidases (PRX33, PRX34) are capable of coordinated ROS production. The apoplastic H_2O_2 can be either perceived by unknown receptors or translocated into cytosol through aquaporin. At the same time, perception of flg22 or O_3 leads to activation of Ca^{2+} channels that generate cytosolic Ca^{2+} influx. Changes in $[Ca^{2+}]_{cyt}$ concentrations are sensed by Ca^{2+} binding proteins (CDPK, CBL/CIPK, CaM), together with activation of MAPK cascades coordinate transcriptional control of gene expression. Such $[Ca^{2+}]_{cyt}$ signals can be also sensed by EF-hands in RBOHD through differential phosphorylation by kinases like CDPK and BIK1. The resulting H_2O_2 feeds back to induce secondary Ca^{2+} influx, which could form a feedback-amplification loop that propagates the Ca^{2+}/ROS signals in both local and systemic tissues. Moreover other internal ROS sources such as chloroplast, mitochondria and peroxisome, contribute to the ROS homeostasis. During specific stresses (i.e. light stress, impaired photorespiration or respiration), the increased ROS level in these organelles are sensed and transmitted to the cytosol and nucleus, leading to altered gene expression. ROS homeostasis is highly integrated with SA signaling and activities of oxygenated lipids (i.e. JA). ICS1 mediates production of SA, which may be transported through the MATE-transporter EDS5 into the cytosol. SA signaling modulates ROS homeostasis and cellular redox state, leading to conversion of cytosolic oligomers NPR1 into monomers. Subsequently, NPR1 monomers are translocated from the cytosol into the nucleus thereby activating SA-mediated transcriptional reprogramming.

1.3 Transcriptional control of defense response induced by apoplastic ROS

Perception of environmental cues leads to dramatic changes in gene expression, executed by DNA-binding TFs and associated regulatory proteins (Zeller et al., 2009; Vaahtera and Brosché, 2011; Rasmussen et al., 2013; Buscaill and Rivas, 2014). Transcriptional regulators induce rapid changes in gene expression to favor defense over other cellular processes such as growth and development (Moore et al., 2011). A ROS burst is a common response to multiple stresses and leads to activation of complex and often interconnected signaling pathways. Ultimately, such signaling cascades frequently results in altered expression of stress-responsive genes. Both forward and reverse genetic approaches have been used to identify TFs and genes involved in gene expression and signal transduction in response to ROS. Extensive expression profiling in a reference plant like Arabidopsis can help explore conserved stress-signaling networks and regulatory mechanisms. A large number of experiments have been performed based on hybridization- or sequence-based approaches, in order to deduce and quantify how the transcriptome changes under a variety of stress and developmental conditions (http://www.ncbi.nlm.nih.gov/geo; https://www.ebi.ac.uk/arrayexpress; http://affymetrix.arabidopsis.info/link_to_iplant.shtml). Compared to the other existing approaches (Tosti et al., 2006), RNA sequencing (RNA-seq) provides a far more precise and sensitive measurement of the abundance of transcripts and their isoforms (Wang et al., 2009). Ideally, transcriptome analysis of ROS signaling with RNA-seq can lead to the identification of crucial regulators and will enable to quantify the effect of each component.

cis-elements typically regulate gene transcription by functioning as binding sites for TFs. TFs and *cis*-elements function in the promoter region of various stress-responsive genes, and overexpression or suppression of TF genes may improve the plant tolerance to multiple stresses (Shanker and Venkateswarlu, 2011). Biochemical and genetic studies of Arabidopsis have identified various TFs that mediate the trade-off between growth and immunity during biotic and abiotic stresses, including AP2/ERF, NAC, TGA/bZIP and WRKY families (Tosti et al., 2006).

The Arabidopsis genome encodes 74 WRKY proteins; studies have indicated that many members of this gene family function as transcriptional activators in plant immune response and response to abiotic stresses. The defining feature of WRKY TFs is the DNA binding domain

(also termed as the WRKY domain) (Ülker and Somssich, 2004). Overexpression and knockdown of specific WRKY TFs' gene expression revealed that several WRKYs integrate signals from different pathways in the defense signaling. For example, MPK4 exists in nuclear complexes with MPK4 SUBSTRATE1 (MKS1)–WRKY33 in absence of pathogens. After infection with *P. syringae* or flg22 treatment, this complex is disrupted, allowing WRKY33 to activate camalexin synthesis and defense gene transcription (Qiu et al., 2008). WRKY70 functions as an activator of SA-induced genes and a repressor of JA-responsive genes, suggesting that WRKY70 acts as a hub for integrating SA- and JA-signaling events during plant defense (Li et al., 2004). In addition, redundancy is often present in this gene family. WRKY70 and WRKY54 co-operate as negative regulators of stomatal closure, osmotic stress, and leaf senescence (Besseau et al., 2012; Jing Li et al., 2013). WRKY46 also cooperates with WRKY54 and WRKY70 in regulation of basal resistance to *P. syringae* (Hu et al., 2012). Substantial evidence indicates that WRKY participate in the interaction between biotic and abiotic resistance through ROS gene-related modulation (Blomster et al., 2011; Jing Li et al., 2013; Brosché et al., 2014; Perez and Brown, 2014).

NAC (for NAM, ATAF1, 2, and CUC2) protein is first identified as RESPONSIVE TO DEHYDRATION 26 (RD26) in Arabidopsis (Aida et al., 1997). Expression of RD26 can be induced by multiple treatments including JA, H_2O_2, and pathogen infection. NAC domain proteins share a conserved N-terminal DNA binding domain that is common to four genes NAM, ATAF1, ATAF2 and CUC2. NAC TFs could regulate many target genes through binding to the CATGTG motif thereby activating transcription in the response to multiple stresses (Nuruzzaman et al., 2013). Overexpression and repression of specific member of NAC gene family is often observed to be associated with oxidative stress, HR, and stress tolerance. Plants overexpressing the stress-induced NAC ATAF1 display a pleiotropic phenotype, including dwarfism, enhanced susceptibility to the necrotrophic pathogen *B. cinerea*, and hypersensitivity to ABA and oxidative stress, suggesting that ROS signaling may be related to ATAF1-mediated stress responses (Nuruzzaman et al., 2013). Likewise, ATAF2 expression appeared to be induced by dehydration, JA, SA, and wound response. Gene expression profiling revealed that overexpression of ATAF2 repressed several *PR* genes, whereas loss of ATAF2 function resulted in increased expression of these genes (Delessert et al., 2005). Intriguingly, overexpression of a H_2O_2-induced NAC TF JUNGBRUNNEN1 (JUB1), greatly delayed senescence, reduced H_2O_2 levels, and enhanced tolerance to various abiotic stresses (Anhui Wu et al., 2012). This suggests a feedback-loop between ROS production and the expression of specific NAC TFs in regulation of plant defenses.

The first plant TF cloned in tobacco, TGA TFs play important roles in in defense responses against biotrophic and necrotrophic pathogens. This family of transcription factors recognizes the TGACG motif, which is found in the promoters of a variety of genes, including *PR1*. The Arabidopsis genome encodes ten TGA TFs falling into five clades. Clade II consists of three closely related TGA TFs, TGA2, TGA5, and TGA6 (Gatz, 2013). They are considered as essential regulators of SAR due to their interaction with the transcriptional coactivator NPR1 in regulation of defense gene expression under inducing and non-inducing SAR conditions. The *tga2-1 tga5-1 tga6-1* triple mutant displays a *npr1*-like phenotype with respect to compromised SAR and abolished *PR-1*-induction after treatment with the SA analogue 2,6-dichloroisonicotinic acid (INA) (Zhang et al., 2003). Mounting evidence has also revealed an essential role for TGA TFs in activation of JA/ethylene induced-defense response. Plants overexpressing GRX480, a mediator of redox regulation, had suppressed *PDF1.2* expression, which was dependent on TGA TFs

(Ndamukong et al., 2007). Conversely, Col-0:*NahG* accumulates 25-fold higher levels of JA and exhibits enhanced expression of *PDF1.2* in response to pathogen infection (Spoel et al., 2003). Similarly, the *npr1* mutant has increased basal and induced levels of *PDF1.2*. In addition, expression of GRX480 is induced efficiently by SA which requires NPR1 and TGA TFs (Ndamukong et al., 2007), suggesting an important role of GRX480 in mediating SA-JA antagonism. Intriguingly, stress-induced ethylene signaling or ACC treatment could make SA-JA antagonism independent of NPR1 (Leon-Reyes et al., 2009). Further studies have revealed that TGA TFs are required for the induction of nearly all ACC-induced genes that are subject to SA suppression via ORA59 (Zander et al., 2014). In addition, TGA2, TGA5, TGA6 appear to regulate specific plant responses to reactive oxylipins (Stotz et al., 2013), suggesting that they have an integral role in JA signaling. Overall, this indicates that TGA TFs act as a molecular link between SA and JA/ethylene signaling.

APETALA2/ethylene-responsive element binding factor (ERF) were originally isolated as proteins that bind to the GCC box (AGCCGCC) present in the upstream region of many JA/ethylene-inducible genes and PR genes (Ohme-Takagi and Shinshi, 1995; Licausi et al., 2013). The AP2/ERF TF family can be divided into two main groups, the Dehydration-Responsive Element Binding-proteins (DREBs) and the ERFs. A wide range of biological functions have been described for ERF family proteins, including pathogen infection, salt stress, osmotic stress, wounding, drought, hypoxia, temperature stress and the stress-related hormones ethylene, jasmonic acid and ABA (Licausi et al., 2013). In contrast, members of DREBs subfamily such as CBFs are known to be induced in response to cold stress and, when ectopically expressed, lead to improved tolerance to freezing (Cook et al., 2004; Yamaguchi-Shinozaki and Shinozaki, 2006). Likewise, overexpression or repression of specific ERF TFs confers tolerance to various biotic stresses. For example, constitutive overexpression of ERF1, ERF2, ERF3, ERF6 and ORA59 induce expression of *PDF1.2* and confers resistance to *B. cinerea* (Berrocal‐Lobo et al., 2002; Pré et al., 2008; Gaiyun Zhang et al., 2009; Meng et al., 2013). Conversely, expression of a repressive form of ERF6 by fusion ERF6 to the ERF-associated amphiphilic repression (EAR) motif leads to hypersensitive to *B. cinerea* (Meng et al., 2013). In addition, ERF TFs are involved in H_2O_2-activated MAPK cascade-triggered oxidative gene transcription in Arabidopsis. ERF6 is phosphorylated by MPK6 and acts as an activator of transcription under oxidative stress (Wang et al., 2013).

1.4 Natural variation occurring in Arabidopsis provides genetic bases for apoplastic ROS signaling

Genetic variation is one additional resource for understanding the molecular mechanisms underlying defense responses and development in plants. As outlined above, mutant analysis in Arabidopsis has been essential in understanding plant stress signaling. However, frequently insertion mutants (e.g. T-DNA insertions) or EMS mutants are limited to loss of function mutants. Genetic differences among Arabidopsis accessions have been found in traits important in responding to drought, cold, salt stress, pathogens, development and O_3 (Mouchel et al., 2004; Brosché et al., 2010; Sutka et al., 2011; Weigel, 2012; Nägele and Heyer, 2013). Identification of the underlying quantitative trait locus (QTL) may pinpoint regulatory mechanisms that could not have been found through mutant analysis. QTL analysis in Arabidopsis often relies on the use of recombinant inbred lines (RILs). However, identification and confirmation of the particular genes or single nucleotide polymorphism (SNP) that cause

the phenotype of interest is still a major challenge with traditional mapping strategies. As a complement, combining genetic mapping and gene expression analysis appears to be a more efficient way to characterize the genetic basis that confers the traits of interest (Chan et al., 2011). For example, gene expression analysis identified a novel negative regulator of freezing tolerance in the QTL regions identified in a RIL population from a reciprocal cross between two Arabidopsis accessions C24 (cold sensitive) and Tenela (Te) (cold tolerant) (Meissner et al., 2013). Coincidently, these two geographically isolated accessions (C24 from Portugal, Te from Finland) also exhibit distinctive phenotype after acute O_3 exposure (300-350 nmol mol^{-1} for 7 h) (Brosché et al., 2010). Such variation in O_3 sensitive between C24 (O_3 tolerant) and Te (O_3 semi-sensitive) makes this RIL population a good genetic tool to uncover potential regulators of apoplastic ROS-induced PCD. Similar to PAMP or MAMP-triggered HR, acute O_3 has to be perceived at the cellular level and relayed to the nucleus, and lead to PCD (Overmyer et al., 2005; Vaahtera et al., 2014), indicating the important role of transcriptomic reprograming in regulation of O_3-induced lesion formation. Measurement of ROS-induced changes in gene expression from a series of O_3 exposure under different time points (0 h, 1 h, 2 h, 4 h, 6 h, 8 h, 24 h) reveal that two hour O_3 exposure could reflect the most informative gene expression about the outcomes in O_3-triggered cell death. Thus RNA-seq analysis of C24 and Te at two hour time point could contribute to identification of signaling components associated with O_3-induced cell death. More importantly, genetic variation in Arabidopsis accessions other than the common laboratory stain Col-0 could provide a promising alternative to gain additional knowledge on the molecular details of O_3 action. Complementary to this RIL population, presence of a huge collection of T-DNA or loss-of-function mutants in Arabidopsis provides a research resources for apoplastic ROS signaling. Disruption of various signaling pathways in the mutants with different sensitivities in response to O_3 treatment, i.e. *dnd1* (O_3 tolerant) and *coi1* (O_3 sensitive), could enable the revelation of genetic basis that confers apoplastic ROS induced-cell death.

2 Aims of the study

The aims of this study were to dissect the signaling components involved in apoplastic ROS-triggered transcriptional reprograming and cell death. Plants exposed to O_3 activate a ROS burst in the apoplast, which is a common response during various biotic and abiotic stresses. Apoplastic ROS signaling is known to be associated with an increase in $[Ca^{2+}]_{cyt}$, alteration of gene expression, modulation of hormone signaling and PCD. However, the way in which these signaling pathways interplay and their contribution to the early apoplastic ROS signaling remain elusive. A combination of forward and reverse genetic tools in Arabidopsis and gene expression analysis were employed to give mechanistic explanations for the responses exhibited.

This dissertation addresses three specific objectives:

1. Unravel the genetic base of the apoplastic ROS-triggered defense response in Arabidopsis accessions beyond the common lab strain Col-0.
2. Elucidate the role of SA, JA, and ethylene signaling and their interaction in regulating O_3 response and cell death.
3. Dissect and quantify the effect of signaling components involved in the early apoplastic ROS signaling.

3 Material and methods

The methods used in this dissertation are described in respective publications (Table 1); plant materials are listed in Table 2; defense related marker genes are listed in Table 3.

Table 1. Methods used in publications I, II, and III. Parentheses indicate that method was contributed or conducted by co-authors.

Methods	Publications
O_3 treatment	I, II, III
Ion leakage	II, (III)
SA treatment	I, II
Microarray analysis	I, II, III
Real-time quantitative PCR analysis	I, II, III
DAB staining	II, III
Trypan blue staining	I, II
Stomata conductance	(II)
Cluster analysis	I, II, III
RNA-seq analysis	(II), (III)
QTL mapping	II
Statistics: One way ANOVA, Two way ANOVA, Linear Mixed model	I, II, III

Table 2. List of Arabidopsis accessions and mutants used in this study. Double or higher order mutants marked with * was generated in this study.

Genotypes	Annotation	Used in	Comments
Col-0		I, II, III	
dnd1/cngc2	DEFENSE NO DEATH 1/ CYCLIC NUCLEOTIDE GATED CHANNEL 2	I	
*dnd1 ein2**	ETHYLENE INSENSITIVE 2	I	
*dnd1 etr1-1**	ETHYLENE RESPONSE 1	I	
*dnd1 mpk3**	MITOGEN-ACTIVATED PROTEIN KINASE 3	I	
*dnd1 mpk6**	MITOGEN-ACTIVATED PROTEIN KINASE 6	I	
*dnd1 ibr5**	INDOLE-3-BUTYRIC ACID-RESPONSE5	I	
*dnd1 rbohD**	RESPIRATORY BURST OXIDASE HOMOLOGUE D	I	
*dnd1 rbohF**	RESPIRATORY BURST OXIDASE HOMOLOGUE F	I	
*dnd1 wrky70**		I	
*dnd1 jin1/myc2**	JASMONATE INSENSITIVE 1	I	
*dnd1 aos/dde2**	ALLENE OXIDE SYNTHASE/ DELAYED DEHISCENCE 2	I	
*dnd1 sid2/ics1**	SALICYLIC ACID INDUCTION DEFICIENT 2/ISOCHORISMATE SYNTHASE 1	I	
*dnd1 npr1**	NONEXPRESSER OF PR GENES 1	I	
*dnd1 eds1**	ENHANCED DISEASE SUSCEPTIBILITY 1	I	
*dnd1 pad4**	PHYTOALEXIN DEFICIENT 4	I	
*dnd1 ald1**	AGD2-LIKE DEFENSE RESPONSE PROTEIN 1	I	
*dnd1 fmo1**	FLAVIN-DEPENDENT MONOOXYGENASE 1	I	
*dnd1 cbp60g**	CAM-BINDING PROTEIN 60-LIKE G	I	
*dnd1 sr1/camta3**	SIGNAL RESPONSIVE 1/CALMODULIN-BINDING TRANSCRIPTION ACTIVATOR 3	I	

*dnd1 agb1 gpa1**		I	
	GTP BINDING PROTEIN BETA 1/ G PROTEIN ALPHA SUBUNIT 1		
*dnd1 era1**	ENHANCED RESPONSE TO ABA 1	I	
*dnd1 rar1-21**	REQUIRED FOR MLA12 RESISTANCE 1	I	
*dnd1 acd5**	ACCELERATED CELL DEATH 5	I	
*dnd1 sid2 ald1**		I	
*dnd1 sid2 eds1**		I	abnormal tumor development
*dnd1 sid2 pad4**		I	
*dnd1 ald1 pad4**		I	
*dnd1 sid2 aos**		I	
*dnd1 sid2 fmo1 **		unpublished	abnormal tumor development
*dnd1 NahG**		unpublished	abnormal tumor development
abi1	ABA INSENSITIVE 1	III	
coi1-16	CORONATINE INSENSITIVE 1	III	
sid2-1		III	
sid2-2		III	
ein2		III	
Nahg	salicylate hydroxylase	III	
*ein2 sid2-1**		III	
*coi1-16 ein2 **		III	
*coi1-16 sid2-1 **		III	
*coi1-16 ein2 sid2-1**		III	
*coi1-16 ein2-1 eds1**		III	
*coi1-16 ein2-1 sid2-1 eds1**		III	
*coi1-16 eds1**		III	
anac017-1	NAC (for NAM, ATAF1, 2, and CUC2)	III	
anac017-2		III	
*anac017-3**		III	
ERF6 4D-5	ETHYLENE RESPONSE FACTOR 4	III	
ERF6 4D-7		III	
ERF6 EAR 65	ETHYLENE RESPONSE FACTOR 6	III	
ERF6 EAR 71		III	
tga2 tga5 tga6		III	
*wrky25 wrky33**		III	
wrky18 wrky40 wrky60		III	
agb1-2		III	
gpa1-4		III	
rbohD		III	
*coi1-16 rbohD**		III	
robhF		III	
*coi1-16 rbohF**		III	
wrky70		III	
*coi1-16 wrky70**		III	
wrky25		III	
*coi1-16 wrky25**		III	
aos		III	
aos ein2		III	
accessions:			
C24		II	
Te		II	
CT101		II	O_3 sensitive RIL candidates
Cvi-0		II	

Table 3. List of defense related marker genes used in this study

Gene name	AGI	Annotation	Used in
bHLH	AT5G56960	BASIC helix-loop-helix (bHLH)	I

CML37	AT5G42380	CALMODULIN LIKE 37	I
LOX4	AT1G72520	LIPOXYGENASE 4	I
PAD3	AT3G26830	PHYTOALEXIN DEFICIENT 3	I
PLA2A	AT2G26560	PATATIN-LIKE PROTEIN 2A	I
PR1	AT2G14610	PATHOGENESIS-RELATED GENE 1	I
SAG21	AT4G02380	SENESCENCE-ASSOCIATED GENE 21	I
WRKY40	AT1G80840		I
WRKY75	AT5G13080		I, III
ZAT10	AT1G27730	ZINC FINGER PROTEIN 10	I
ZAT12	AT5G59820	ZINC FINGER PROTEIN 12	I
AOX1a	AT3G22370	ALTERNATIVE OXIDASE 1a	III
ARGOS	AT3G59900	AUXIN-REGULATED GENE INVOLVED IN ORGAN SIZE	III
ARR5	AT3G48100	ARABIDOPSIS THALIANA RESPONSE REGULATOR 5	III
CRK9	AT4G23170	CYSTEINE-RICH RLK (RECEPTOR-LIKE PROTEIN KINASE)9	III
CRK39	AT4G04540	CYSTEINE-RICH RLK (RECEPTOR-LIKE PROTEIN KINASE)39	III
ERF6	AT4G17490	ETHYLENE RESPONSIVE ELEMENT BINDING FACTOR 6	III
GRX480	AT1G28480	GLUTAREDOXIN 480	III
IDA	AT1G68765	INFLORESCENCE DEFICIENT IN ABSCISSION	III
LOX4	AT1G72520	LIPOXYGENASE 4	III
ODX/DIN11	AT3G49620	DARK INDUCIBLE 11	III
ORA59	AT1G06160	OCTADECANOID-RESPONSIVE ARABIDOPSIS AP2/ERF 59	III
RBOHD	AT5G47910	RESPIRATORY BURST OXIDASE HOMOLOGUE D	III

4 Results and Discussion

Forward and reverse genetic tools in Arabidopsis provide enormous possibilities to dissect the signaling pathway(s) activated by specific stresses. Naturally-occurring variation of O_3 sensitivity among different Arabidopsis accessions provided genetic tools to identify novel regulators involved in apoplastic ROS signaling. To gain further insight into the genetic basis of apoplastic ROS-triggered leaf damage, a RIL population was generated by crossing between the O_3-tolerant accession C24 and the O_3-sensitive accession Tenela (Te). This RIL population was employed to perform quantitative trait loci (QTL) mapping for apoplastic ROS-triggered cell death. In addition, QTL analysis of a F2 population generated from C24 and Te and a backcrossing population generated from C24 and a sensitive RIL CT101 were performed to validate the accuracy of QTLs obtained from analysis of RIL population (II). Through a combination of QTL and RNA-seq analyses in response to apoplastic ROS treatment, three major QTL regions containing several potential candidate genes were identified (II). Gene expression data suggested that constitutive activation of SA mediated signaling was found to be responsible for tolerance of C24 (II). Interaction between the hormones SA, JA and ethylene optimizes the responses to abiotic and biotic stresses. Although early studies revealed that the SA, JA and ethylene participated in regulation of O_3-induced cell death formation and changes in gene expression, little is known about how these three hormones interact with early apoplastic ROS signaling. In this dissertation, a combination of single, double and triple mutants was employed to assess the contribution of SA/JA/ethylene on early apoplastic ROS signaling. Furthermore, mutants with altered SA-dependent and independent signaling, TFs, or with a previously described role in cell death or defense against pathogens were used (Table 2). Microarray analysis of the O_3 tolerant LMM *dnd1* and RNA-seq analysis *of coi1-16 ein2 sid2* deficient in SA/JA/ethylene signaling were performed to quantify the effect of the three hormones on the early apoplastic ROS signaling (I, II, III). The triple mutant *tga2 tga5 tga6* was used in RNA-seq to evaluate the role of these TFs in early apoplastic ROS signaling (III). Further gene expression analysis was performed using real-time reverse-transcriptase quantitative PCR (qPCR) with marker genes selected from both array data and RNA-seq data (Table 3).

4.1 The value of mutants versus natural variation in dissection of apoplastic ROS signaling

The existence of the complete genome sequence and a huge collection of genetic resources from Arabidopsis have enabled functional analysis of individual genes. Genetic tools include disruption of proper gene expression, such as T-DNA or transposon insertion, RNA interference (RNAi), and as an alternative overexpression of the gene of interest. Characterization of phenotypes of interest and the associated genes with subsequent effect on the other genes have often relied on using loss-of-function mutants. The availability of large collection of indexed T-DNA mutants allows researchers to rapidly design studies related to their genes of interest. Besides genomics and reverse-genetics approaches, phenotype-driven forward genetics using mutagenized populations has been among the most successful approaches to reveal novel genes/alleles and for understanding signaling pathways. A complementary approach is to use the natural variation among different Arabidopsis accessions. This could

allow us to identify novel genes or alleles not present in the common laboratory stain Col-0. In this dissertation, both mutant analysis and natural variation were employed to dissect the signaling components involved in the apoplastic ROS signaling.

A previous study revealed extensive natural variation in O_3 sensitivity among Arabidopsis accessions (Brosché et al., 2010). The accession C24 was identified as tolerant to O_3, whereas Te was considered a semi-O_3-sensitive accession. To investigate the genetic basis of O_3-triggered PCD, a RIL population generated from reciprocal crosses between C24 and Te was used for quantitative trait loci (QTL) mapping. Furthermore, a backcross-population (from a cross between the O_3-sensitive RIL CT101 and C24) and F2 population (from a cross between C24 and Te) were employed to confirm the accuracy of the QTL regions. QTL analysis revealed three major QTL regions responsible for apoplastic ROS-triggered cell death (II). However, the three QTL regions were rather large making identification of the responsible gene and the SNPs underlying the observed O_3 sensitivity a challenging and time-consuming task. In a complementary experiment, a combination of QTL and RNA-seq analysis were employed to elucidate the potential signaling components involved in O_3-induced changes in gene expression and cell death. Although detailed investigation of gene expression might be insufficient to confirm the causal genes that confer the altered defense and execution of cell death, such analysis could still narrow down the number of the candidate genes. In the C24-Te QTL mapping, gene expression analysis suggested that genes related to antioxidant biosynthesis and recycling, transcription regulation and SA biosynthesis could be involved in regulation of O_3 sensitivity (II). Another challenge of using O_3-triggered PCD-driven QTL mapping in this RIL population was to avoid false-positive and false-negative scoring during phenotypic identification of leaf damage due to Te alleles contributing to sensitivity being of small effect. Hence, a backcross and F2 populations were used to validate the location of the QTLs in response to O_3.

To complement the analysis of natural variation, using mutants in the widely studied accession Col-0, especially the loss-of-function mutants, enables functional analysis of individual genes involved in apoplastic ROS signaling. Generation of double or triple mutants allows the investigation of the interaction between different signaling pathways. The effect (e.g. phenotype) of essential mutants in a given signaling pathway can therefore be clearer in comparison with QTLs. Such mutant-associated analysis is often combined with transcriptome profiling under different conditions to identify interactive signaling components and target genes in signaling network. In this study, both O_3-tolerant and sensitive mutants were employed to unravel the essential signaling components in apoplastic ROS signaling (Table 2). A combination of double and triple mutants were generated from crossing among the SA deficient mutant *sid2*, the JA-insensitive mutant *coi1-16* and the ethylene-insensitive mutant *ein2* to study the combined role of these hormones signaling during early apoplastic ROS signaling.

4.2 The effect of hormone signaling and other signaling components on apoplastic ROS induced transcriptome reprogramming

4.2.1 SA signaling plays a dual role in regulation of apoplastic ROS signaling

A stress-induced ROS burst is known to be associated with alteration of gene expression and activation of SA/JA/ethylene signaling (Kangasjärvi et al., 2005). Although early studies revealed that the O_3-triggered production of H_2O_2 and initiation of cell death were in direct proportion to the accumulation of SA in O_3-sensitive genotypes (Rao et al., 1997; Rao and Davis, 1999; Rao et al., 2000; Overmyer et al., 2003), little was known about the early signaling events underlying this finding. Previous analyses indicated that the LMM *dnd1* with constitutive activation of SA signaling exhibited attenuated responses to both acute O_3 and flg22 treatment (Overmyer et al., 2005; Mersmann et al., 2010; Wrzaczek et al., 2010). Global gene expression analysis has previously been performed on *dnd1/cngc2* (Chan et al., 2008). However, since *dnd1* displayed different phenotypes dependent on growth conditions, a new array analysis was performed with *dnd1* to identify: (a) which signaling pathways were activated in this mutant under the growth conditions used in this study (I); (b) to identify suitable marker genes for the subsequent qPCR experiment (Table 3). The genes significantly regulated by *dnd1* were subjected to Bayesian hierarchal cluster analysis with several experiments including LMM mutants, senescence experiments, hormone treatments, and pathogen treatments (I). The cluster analysis revealed that constitutive activation of defense responses was shared between *dnd1* and these defense-related experiments (I), indicating such defense-related genes could be commonly activated in response to different stresses.

To examine the biological significance of constitutive SA signaling in relation to early apoplastic ROS signaling triggered by O_3, Arabidopsis mutants with enhanced (*dnd1*, *cim7*, *cim13*, *lht1*, *siz1-2*, *sr1/camta3*) and deficient SA signaling (*sid2*, *ald1*, *eds1*, *pad4*) were subjected to two-hours of acute O_3 exposure and then analysis of expression of selected defense marker genes from the *dnd1* array analysis was performed (I, Table 3). In the absence of stress, all constitutive defense mutants exhibited elevated transcript abundance for the selected marker genes in contrast to wild-type Col-0 (I). Likewise, RNA-seq analysis of the O_3 tolerant accession C24 revealed that SA signaling was constitutively activated in fresh air (II). Intriguingly, no induction of the SA marker gene *PR1* was evident in response to O_3 exposure, suggesting that no substantial accumulation of SA was induced by O_3 within two hours (I). The two-hour exposure to O_3 led to significant accumulation of most of the selected defense and cell death related genes in both O_3-sensitive and O_3-tolerant genotypes, but the O_3 induction was less pronounced in the constitutive defense mutants and the accession C24 (I, II). In addition, the O_3 response was quantitatively higher in the mutants lacking SA such as *sid2* and *ald1*, suggesting that basal levels of SA are required for activation of a response that could attenuate apoplastic ROS signaling (I). Introduction of *sid2* into *dnd1* partially restored its responses to O_3, whereas simultaneous removal of the SA-related regulators *EDS1* or *ALD1* with *SID2* resulted in complete recovery of the response to O_3 in *dnd1* (I). Thus, each regulator of SA signaling contributes certain effect(s) on the apoplastic ROS signaling. To gain further insight into the effect of SA on the apoplastic ROS signaling, Col-0 was sprayed with 0.3 mM and 1 mM SA prior to O_3 treatment (I). SA pretreatment at both concentrations attenuated the induction of defense-marker genes expression in response to O_3 except *PR1*, *PR2* and flg22 responsive gene *FRK1* (*FLG22-INDUCED RECEPTOR-LIKE KINASE 1*) (I). In contrast to the other tested genes, expression of *FRK1* was synergistically increased by combined SA and the O_3 treatment (I). Overall, these data demonstrate that SA signaling could synergistically and antagonistically regulate apoplastic ROS signaling and activate defense related genes.

4.2.2 The role of JA and ethylene signaling in the apoplastic ROS signaling

The synergistic interaction between JA and ethylene is best studied in the response of plants to necrotrophic pathogens (Pieterse et al., 2012). Early studies revealed that JA acts as a negative regulator of lesion development during acute O_3 exposure, whereas ethylene positively contributed to lesion formation (Overmyer et al., 2003). However, very often, these studies used mutants such as *coi1* and *jar1* to analyze the signaling role of JA, whereby the effect of other oxylipins such as OPDA could not be excluded. To examine whether JA signaling contributes to the early apoplastic ROS signaling, mutants with impaired biosynthesis (*aos*) and JA perception (*coi1-16*) were exposed to two-hour O_3 treatment and then the transcript abundance of selected defense marker genes was quantified (I, III). After a two-hour O_3 exposure, the majority of the selected defense marker genes exhibited similar induction of expression between Col-0 and the two mutants, except *ARR5*, *ERF6*, and *IDA*. In contrast to *aos*, the increased of expression of *ERF6* and *IDA* was smaller in *coi1-16*, and increased of expression of *ARR5* was higher in *coi1-16*. Furthermore, the differential induction of *ERF6*, *IDA* and *ARR5* implied that two oxylipins JA and OPDA might contribute different effect to the apoplastic ROS signaling (I, III).

Similar to *coi1-16* single and related double and triple mutants, JA signaling seemed to be impaired in the O_3-tolerant genotype C24 but not in the O_3-sensitive genotype Te and CT101 (II). For example, a highly O_3-inducible gene *ODX* (also known as *DARK INDUCED11* (*DIN11*)) was induced in a COI1- and DDE2-dependent manner even though JA-Ile levels were not increased (Köster et al., 2012). *ODX* expression was low in *coi1-16 ein2*, *coi1-16 ein2 sid2* and C24 under both fresh air and O_3 condition; in contrast expression of this gene in Te/CT101 and the O_3-tolerant accession Col-0 was strongly induced by O_3 (II, III). This could indicate a role for JA signaling even when the amounts of JA-Ile are low.

There was no significant effect of ethylene alone on changes in O_3-regulated gene expression; neither in a mutant with constitutive activation of ethylene signaling (*ctr1*) nor in a mutant with impaired ethylene signaling (*ein2*) (I, II). An exception to this rule was expression of *ARGOS*, where no effect of O_3 on the changes in gene expression of *ARGOS* was observed in mutants with *ein2* background. Instead it appeared that the role of ethylene was to cooperate or modify the ability of JA to regulate the O_3-induced gene expression. For example, the induction of expression of *WRKY75* in *coi1-16 ein2* was significantly weaker than *coi1-16* (III). Similarly, *coi1-16 ein2* had enhanced induction of *IDA* in comparison to the *coi1-16* single mutant (III). Altogether, JA signaling could function as both a positive and negative modifier in regulation of the specific genes of the early-apoplastic ROS signaling network, where some part of JA-triggered signaling could be enhanced when ethylene signaling is impaired. In addition, ethylene signaling could cooperate with SA signaling in regulation of apoplastic ROS signaling. Both enhanced ROS production and SA accumulation were found in the constitutively-activated ethylene mutant *eto1* and *eto3* after O_3 treatment (Rao et al., 2002). By contrast, flg22-induced ROS production was impaired in the *ein2* mutant (Mersmann et al., 2010), suggesting that ethylene signaling is required for apoplastic ROS signaling.

4.2.3 The contribution of SA, JA, and ethylene-dependent signaling and TFs to the apoplastic ROS signaling

Tightly coordinated regulation of different signaling pathways is required to fine-tune the

defense response during many stresses. As noted above, each hormone-signaling pathway specifically contributes to certain parts of the early-apoplastic ROS signaling network. In order to quantify the effect of the three hormones on the early-apoplastic ROS signaling, a transcriptome analysis of Col-0 and *coi1-16 ein2 sid2* triple mutant was performed with RNA-seq in both fresh air and two-hour O_3 conditions. Consistent with the qPCR results, a substantial number of O_3-regulated genes were similar between Col-0 and *coi1-16 ein2 sid2*. SA, JA and ethylene-dependent signaling contributed to about 30% of the early apoplastic ROS-regulated changes in gene expression (III). The O_3-regulated genes were then subjected to gene ontology (GO) enrichment analysis. Given the high overlap between Col-0 and *coi1-16 ein2 sid2* regulated genes, also the enriched GO categories after O_3 treatment were also common to the two genotypes. However, a strong genotypic effect was apparent in the comparison of *coi1-16 ein2 sid2* with Col-0 in the absence of O_3 (control conditions) (III). Out of 769 genes significantly altered in *coi1-16 ein2 sid2* in comparison with Col-0 in fresh air, over 600 genes exhibited significantly decreased expression and enrichment in GO categories SA, JA, ethylene and cell death (III). Interestingly the GO category cell death was also significantly enriched among the genes with increased O_3 induction in *coi1-16 ein2 sid2* (III). This indicates that expression of the genes involved in cell death was suppressed in *coi1-16 ein2 sid2* in the absence of O_3, whereas after the O_3 treatment the induction of cell death genes were more pronounced than in *coi1-16 ein2 sid2* than in Col-0. This could be explained by the existence of putative negative regulator of cell death that is expressed to low levels in *coi1 ein2 sid2*; in the O_3 treatment this negative regulator could not suppress expression of cell death related genes, leading to the observed enrichment of cell death related genes with higher expression in O_3 treated *coi1-16 ein2 sid2*.

In addition to GO analysis, promoter enrichment analysis was performed to understand which TFs might be involved in regulation of early O_3 signaling. Similar to the high overlap in GO categories, multiple promoter elements were also commonly enriched in both genotypes after the two-hour O_3 treatments, including the GCC-box, NAC, W-box, TGA and others (III). Such cis-elements can be bound by many TFs in each TF family, something that could mask the contributions of specific TF from the same TF family. In order to assess the effects of TFs on the early apoplastic ROS-induced changes in gene expression, several TF mutants from the WRKY, NAC, ERF, and TGA TF families were selected based on their important roles in various stress-induced defense responses including O_3. For example, from the top 30 highly O_3-inducible TFs (more than 50-fold), ten were ERFs and eight were WRKYs (III).

The role of ERF TFs in the apoplastic ROS signaling

The GCC-box, bound by ERF TFs, was especially enriched in Col-0 in response to apoplastic ROS treatment but not in *coi1-16 ein2 sid2* (III), suggesting that the ERF TFs could involve in apoplastic ROS-driven hormone signaling. Microarray analysis revealed that JA/ethylene-responsive genes and genes containing the GCC-box promoter element were highly induced in plants overexpressing ERF6. This suggests that ERF6 may act as positive regulator of JA-mediated signaling (Moffat et al., 2012). However, a clear redundancy was observed between ERF TFs in disease resistance. For example, no obvious effect on the susceptibility to *B. cinerea* was evident in single mutants of *erf5* or *erf6*, whereas the double mutant *erf5 erf6* had significantly increased susceptibility to *B. cinerea*. A trick to overcome genetic redundancy among TFs is to add a repressor domain and convert a positive regulator to a negative regulator. This can be done through the ERF-associated amphiphilic repression (EAR) motif (Hiratsu et al.,

2003). Arabidopsis expressing ERF6-EAR repressed expression of *B. cinerea*-induced defense genes that led to hypersensitivity to *B. cinerea*. Several studies have documented an important role for ERF6 in triggering the expression of specific ROS response through the GCC-box (Meng et al., 2013; Wang et al., 2013). In this study both the constitutively-active and the repressive EAR forms of EFR6 were selected to assess their effect on apoplastic ROS regulated gene expression. Consistent with RNA-seq analyses (Meng et al., 2013), plants expressing a constitutively-activated version of ERF6 had enhanced expression of all selected defense marker genes in the absence of stress (with the exception of *ARGOS*) (III). Likewise, mutants with constitutively activated SA defense response (I), overexpressing *ERF6* could trigger plant acclimation and raise the threshold of tolerance to apoplastic ROS treatment (I, III). In contrast, the constitutively repressive ERF6-EAR exhibited significantly decreased expression of some defense marker genes including *ODX*, *ORA59*, *RBOHD* and *WRKY75* in fresh air but the O_3-induced changes in expression of the marker genes were intact (III), indicating that other TFs could regulate the apoplastic ROS response of these genes.

The role of NAC TFs in the apoplastic ROS signaling

The NAC motif, bound by ANAC TFs, was significantly enriched among apoplastic ROS-response genes (III). NAC TFs have been shown to regulate multiple stress responses including ROS homeostasis, HR, activation of defense-related genes, and accumulation of hormones (Nuruzzaman et al., 2013). However, a single ANAC TF often responds to multiple stresses, and several of ANAC TFs may redundantly participate in the regulation of similar processes as negative or positive regulators. Several recent studies have revealed the role of TFs from this family, such as *ANAC013*, *ANAC017* and *JUB1* in regulation of H_2O_2 accumulation and mitochondrial retrograde signaling (Anhui Wu et al., 2012; De Clercq et al., 2013; Ng et al., 2013). However, the specific and precise transcriptional outputs of each NAC TF in the early apoplastic ROS signaling remain unknown. ANAC017 could directly bind to the promoter of ALTERNATIVE OXIDASE (*AOX1a*), a protein that alters mitochondrial ROS production by providing an alternative electron sink (Cvetkovska and Vanlerberghe, 2012, 2013). Furthermore, a previous study revealed that the function of ANAC017 was required for proper expression of H_2O_2-induced changes in genes expression (Ng et al., 2013). To further elucidate the potential role of ANAC TFs in apoplastic ROS signaling, two loss-of-function alleles (*anac017*-1, *anac017-3*) and one gain-of-function allele (*anac017-2*) were selected and used for qPCR analysis (III). With the same marker genes (Table 3, III), only O_3-induced expression of *AOX1a* was reduced in the gain-of-function *anac017-2* (III). However since induction of all marker genes by apoplastic ROS treatment was intact in the loss-of-function *anac017* alleles, it was most likely that ANAC017 was not involved in regulation of *AOX1a* or other marker gene expression via apoplastic ROS. Moreover, ANAC017 was required for regulation of 87% of H_2O_2 responsive genes (Ng et al., 2013), whereas in apoplastic ROS signaling none of tested marker genes were altered in loss of function *anac017* mutants (III). This result indicates that apoplastic ROS signaling activated by O_3 could employ different signaling pathways compared to H_2O_2 and mitochondrial retrograde signaling.

The role of WRKY TFs in the apoplastic ROS signaling

Another cis-element; the W-box bound by WRKY TFs, was significantly enriched in the promoters of the apoplastic ROS-responsive genes (III). Early studies revealed that WRKY were

induced by both biotic and abiotic stresses and regulate various aspects of development and cell death-associated ROS signaling (Gadjev et al., 2006; Jiang and Deyholos, 2009; Jing Li et al., 2013). To examine the significance of specific WRKY TFs in the apoplastic ROS signaling, several WRKY TF mutants were selected for further analysis due to the high induction of their corresponding genes by O_3 treatment, including WRKY33, WRKY40, and WRKY60. Single WRKY mutants (*wrky18*, *wrky40*, and *wrky60*) have been shown to exhibit little or no alterations in the response to hemibiotrophic and necrotrophic pathogens but increased susceptibility was observed in double or triple mutants *wrky18 wrky40*, *wrky18 wrky60*, and *wrky18 wrky40 wrky60* (Xu et al., 2006; Brotman et al., 2013). Furthermore WRKY40 and WRKY63 appeared to be involved in regulating the expression of stress-responsive genes encoding mitochondrial and chloroplast protein (Jiang and Deyholos, 2009; Van Aken et al., 2013). Another WRKY, WRKY70, appeared to have the opposite effects on SA and JA signaling, thereby providing a cross-link or convergence point for the antagonistic interaction between these signaling pathways (Li et al., 2004). WRKY25 and WRKY33 have been extensively studied for their roles in pathogen responses, salt stress, and heat stress (Jiang and Deyholos, 2009; Li et al., 2009; Birkenbihl et al., 2012). To overcome redundancy among closely related WRKYs, the *wrky25 wrky33* double mutant and *wrky18 wrky40 wrky60* triple mutant were used in qPCR to examine the contribution of WRKY TFs to apoplastic ROS signaling. No striking difference in the response to O_3 treatment was evident in the *wrky* mutants using the same marker genes (Table 3), (III). Thus, either these WRKYs were insignificant in O_3 signaling, or further redundancy among WRKYs may mask their contribution or a wider selection of marker genes needs to be tested.

Mounting evidence from microarray studies has revealed an over-representation of W-box elements within the promoters of many genes that are involved in biotic and abiotic stresses (Ülker and Somssich, 2004; Gadjev et al., 2006; Wang et al., 2006; Blomster et al., 2011; Niu et al., 2012). To explore the specificity of the W-box, a list of putative target genes was selected from O_3-regulated genes that have at least three W-boxes in their 500 bp promoter region (III). Together with LMM mutants (*acd11*, *lht*, *siz1*, *mkk1 mkk2*), the available gene expression data for different WRKY mutants under various treatments were obtained from public databases and subjected to Bayesian hierarchical clustering (III). Consistent with the qPCR result, no effect on the selected list of genes that contain at least three W-boxes was found in either single knockout-mutant or overexpressor in the absent of stresses (III). Strikingly, this list of genes appeared to be induced by multiple stresses instead of specific stress. All experiments, including different pathogen infections, Benzothiadiazole (BTH) treatment (SA-analog), and LMM, caused similar trends of altered gene expression in different WRKY mutants with exception of high light treatment. Two groups of genes exhibited opposite induction in the high light experiment (III). Given that ROS production can be initiated from different subcellular compartments by high-light stress (chloroplast) and O_3 treatment (apoplast), plants could spatiotemporally activate or suppress the same signaling pathways in response to various stresses.

The role of TGA TFs in the apoplastic ROS signaling

The TGA motif (TGACG) and the longer H_2O_2-regulated NRXE2 (TGACGTCA) (Geisler et al., 2006) were both enriched among apolastic ROS-regulated genes (III). The three closely related Arabidopsis basic leucine zipper (bZIP) transcription factors TGA2, TGA5 and TGA6 are the most-studied members of this TF family (Ndamukong et al., 2007; Gatz, 2013; Stotz et al., 2013). Experiments with the *tga2 tga5 tga6* triple mutant unraveled the essential role of these

subclass II TGA factors in the establishment of SAR, interactions between SA signaling and JA/ethylene signaling, oxylipin and JA signaling (Zhang et al., 2003; Stotz et al., 2013; Zander et al., 2014). The glutaredoxin GRX480 was previously described as one of the hormone-crosstalk modulators, which could be recruited by TGA TFs to integrate at the ORA59 promoter and negatively regulate JA/ethylene signaling (Ndamukong et al., 2007; Zander et al., 2012; Zander et al., 2014). Of the tested markers genes (Table 3), only *GRX480* and *ODX* had reduced transcript abundance, reemphasizing the essential role of TGA2, TGA5 and TGA6 in the regulation of SA and JA signaling in response to the apoplastic ROS treatment (III). To investigate the full role of the TGA TFs and their contribution to the early apoplastic ROS signaling, a RNA-seq transcriptome analysis of *tga2 tga5 tga6* was performed. Similar to the *coi1-16 ein2 sid2* triple mutant, about 80% of O_3-regulated genes displayed similar regulation in *tga2 tga5 tga6* and Col-0 (III). Thus, most of the early O_3-signalling was not dependent on TGA2, TGA5 and TGA6. The major genotypic effect of *tga2 tga5 tga6* in fresh air was seen in genes with increased expression in the GO category JA and decreased expression in the GO category SA. As expected, the TGA promoter element was significantly enriched in genes with lower expression in *tga2 tga5 tga6* (III). Overall, these data suggested that TGA2, TGA5 and TGA6 act as positive regulators of SA signaling and negative regulators of JA signaling.

In this study, several different genotypes with various O_3 responses or sensitivities were used in RNA-seq analysis (O_3 tolerant: C24, Col-0 and *tga2 tga5 tga6*; O_3 sensitive: Te, CT101, Cvi-0 and *coi1-16 ein2 sid2*). To explore if there could be some genotypic differences in the regulation of cell death-related genes, all RNA-seq experiments were combined in Bayesian hierarchical clustering of genes from the GO category cell death (Figure 2). Considerable similarity among all genotypes was apparent from the cluster analysis, but with lower transcript abundance in the tolerant accession C24, which is consistent with less transcript abundance in the mutants (*dnd1*, *cim7*, *cim13*, *lht1*, *siz1-2*, *sr1/camta3*) with constitutively activated defense response (I). The overall similarity in regulation of O_3-induced expression of cell death-related genes indicates that this is a robust response that cannot easily be altered through mutants or genetic variation. This could suggest the existence of several parallel signaling pathways, thus a single mutant is not sufficient to alter the O_3 response. Indeed, not even inactivation of SA, JA and ethylene in *coi1 ein2 sid2* was enough to substantially alter this response. All together, the qPCR and RNA-seq experiments have shed light on the molecular mechanism underlying the antagonism and synergism among hormones signaling. Furthermore, the contribution of TGA2, TGA5 and TGA6 was quantified in the early apoplastic ROS signaling response.

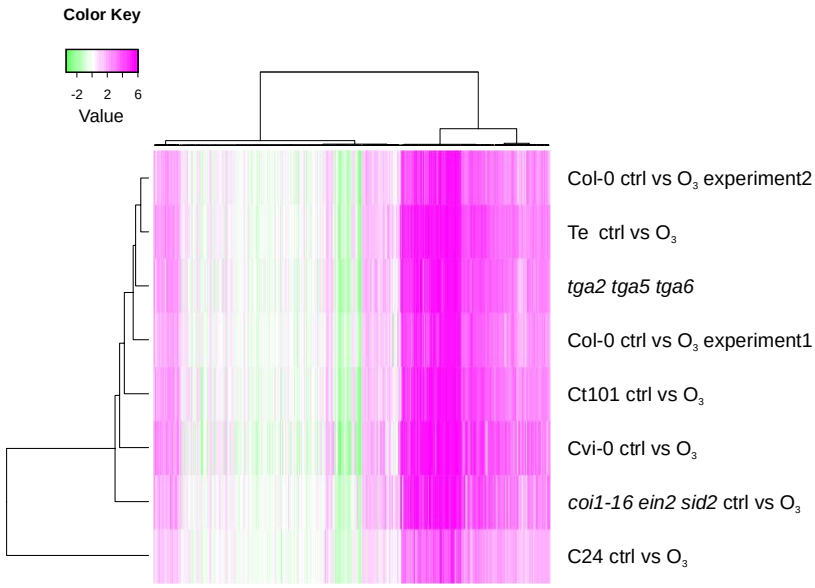

Figure 2. Bayesian hierarchical clustering of genes from the GO category cell death in C24, Te, CT101, Col-0, *coi1-16 ein2 sid2* and *tga2 tga5 tga6* control in comparison with O_3. Values are mean of $\log_2$ ratio of the treatment and control expressions. Magenta and green indicate increased and decreased expression compared with untreated plants.

4.3 The role of hormone signaling and apoplastic ROS in regulation of cell death

Global gene expression profiling with RNA-seq and microarray studies revealed that multiple cooperating signaling pathways participated in regulation of the apoplastic ROS-induced defense responses. Apart from transcriptome reprograming, apoplastic ROS is known to cause lesion formation in sensitive genotypes. During biotic stresses, HR is a typical defense strategy for maximizing survival rate and escaping from pathogen attack. HR involves activation of Ca^{2+} signaling, ROS formation, MAP kinase signaling cascades and activation of SA/JA/ethylene signaling. Importantly, the cell death in response to O_3 is similar to HR and PCD (Overmyer et al., 2005; Vainonen and Kangasjärvi, 2014). *dnd1* is a mutant with constitutively activated defenses that does not display HR in response to infection with an avirulent pathogen (Clough et al., 2000). *dnd1* is also a conditional LMM that displays spontaneous lesions under certain growth conditions. Plants without functional DND1/CNGC2 lack both cell membrane Ca^{2+} influx and ROS production (Mersmann et al., 2010; Chin et al., 2013). The *dnd1* mutant exhibited attenuated response to apoplastic ROS-induced changes in gene expression (I). Differential O_3 sensitivity also occurred among Arabidopsis accessions, i.e. C24 and Col-0 were O_3-tolerant accessions whereas Te and Cvi-0 were more sensitive to O_3. These forward and reverse genetic tools were therefore employed in this study, to dissect the various signaling components underlying the regulation of PCD (Table 3).

4.3.1 The role of SA in regulation of cell death

Like many other LMMs, *dnd1* displays growth retardation, elevated levels of SA and spontaneous cell death. To unravel the elements contributing to this pleiotrophic phenotype, the *dnd1* mutant was crossed with various other mutants deficient in different hormones signaling, MAP kinases, TFs, ROS biosynthesis or mutants with a prescribed role in cell death or defense against pathogens (I).

Regulators involved in SA biosynthesis and SA signaling were especially able to regulate the extent of cell death. Depletion of SA biosynthesis by the introduction of *sid2* into *dnd1* led to decreased cell death and enlarged rosette size (I). Similarly, SA was shown to regulate cell death and leaf senescence, since restored growth was observed by introducing *sid2* or salicylate hydroxylase (*NahG*) into several LMMs, such as *acd6*, *syp121 syp122*, and *atg5* (Rate et al., 1999; Zhang et al., 2007; Zhang et al., 2008; Yoshimoto et al., 2009; Ng et al., 2011). Thus, SA levels could act as a central hub in determining life and death in plants. Likewise, this is supported by introduction of the *NahG* transgene into O_3-hypersensitive accession Cvi-0, where the resulting Cvi-0:NahG has significantly decreased cell death (Rao et al., 2000). In addition, genetic analysis of single mutants with decreased SA-accumulation indicated that *ALD1*, *FMO1*, *EDS1* and *PAD4* redundantly contributed to the development of cell death in *dnd1* (I). EDS1 and its closely related partner PAD4 are essential regulators of basal immune response and SA-dependent signaling (Rietz et al., 2011). FMO1 positively regulates the EDS1 pathway in a SA-independent manner (Bartsch et al., 2006). In addition, EDS1 has been shown to form a complex with both pathogen effectors as well as R proteins (TIR-NB-LRR), and the translocation of such complexes between the cytoplasm and nucleus is required for full activation of local resistance (Heidrich et al., 2011). The *dnd1 ald1 pad4* triple mutant also displayed improved growth compared to *dnd1 ald1* and *dnd1 pad4* double mutants (I); such an additive effect was also evident in *acd6-1 ald1-1 pad4* (Ng et al., 2011).

To further examine the additive contribution of SA and each individual signaling component to the initiation of cell death, several triple mutants were generated through crossing the *dnd1 sid2* double mutant with *dnd1 fmo1*, *dnd1 ald1*, and *dnd1 eds1* double mutants. The resulting triple mutants *dnd1 sid2 fmo1*, *dnd1 sid2 ald1* and *dnd1 sid2 eds1* displayed reduced cell death and improved growth compared to the double mutants (I). Likewise, the addictive effect of *ald1* and *fmo1* on the suppression of cell death was also present in the *acd6* and *syp121 syp122* mutants (Zhang et al., 2008; Ng et al., 2011). *ALD1* and *FMO1* were previously reported to be associated with Pip biosynthesis or signaling; this non-protein amino acid was proposed to function as a systemic-defense signal together with ROS and SA in the regulation of SAR (Návarová et al., 2012).

The aim of generating all the double and triple mutants listed above was to reduce or remove SA signaling. To test the role of enhanced SA signaling on *dnd1* phenotypes, the LMMs *acd5* and *sr1* were crossed into *dnd1* (I). The resulting *dnd1 acd5* and *dnd1 sr1* had massively increased amount cell death. Thus, strongly increased SA signaling led to extensive cell death. Furthermore, this demonstrated that *dnd1* was most likely not in the same signaling pathway as *acd5* or *sr1*, given the additive cell death phenotype of the double mutants.

However, elevated SA levels are not always associated with cell death. For example, the *cim2*

and *cim3* mutants displayed elevated total and free SA concentration without incurring cell death (Ryals et al., 1996). Likewise, the Arabidopsis accession C24 exhibited constitutively activated SA-dependent signaling and broad resistance to many pathogens without developing cell death (II)(Lapin et al., 2012). Higher basal levels of SA could reduce apoplastic ROS induction of gene expression (I). To test the functional relevance of this observation in relation to cell death, two different concentrations of SA were sprayed 16 hours prior to O_3 exposure on both sensitive and tolerant accessions (II). In the O_3-sensitive accessions Te and Cvi-0, a high dose of SA (1 mM) pretreatment substantially decreased apoplastic ROS-induced H_2O_2 accumulation and cell death. In contrast, a low concentration of SA was insufficient to alter O_3-triggered cell death. Overall these findings indicated an essential role for SA in signaling and regulating ROS homeostasis and in the development of cell death, but also show that the role of SA is dose and genotype-dependent.

4.3.2 The role of JA, ethylene and TFs in the regulation of cell death

The balance of the hormones SA and JA is thought to equip the plant with powerful regulatory capacity to fine-tune its defense response to biotic and abiotic stresses (Pieterse et al., 2012). Analysis of mutants deficient in JA signaling revealed a significant effect of JA on the modulation of O_3-induced H_2O_2 content and cell death (Rao et al., 2000). Ethylene is often associated with JA signaling and affects the outcome of JA response. In order to quantify the contribution of JA and ethylene signaling to the regulation of apoplastic ROS-induced cell death, the *coi1-16* mutant with impaired JA signaling were crossed with mutants involved in SA signaling, ethylene signaling, ROS production, heterotrimeric G protein signaling, and WRKY TFs (Table 2). Methyl jasmonate (MeJA) pretreatment of Cvi-0 led to decreased SA levels and completely abolished O_3-triggered cell death, indicating an antagonistic relationship between JA and SA signaling in controlling the extent of O_3-induced cell death (Rao et al., 2000). This raises the possibility that *coi1* and *jar1* were sensitive to O_3 due to their lack of inhibition of SA signaling. However, abolishing SA biosynthesis by the introduction of *sid2* in *coi1-16* did not alter the extent of cell death induced by apoplastic ROS treatment, indicating such antagonism might not be responsible for development of cell death (III). Ethylene was shown to be required for a flg22-triggered ROS burst, suggesting that ethylene could act as positive regulator in ROS production (Mersmann et al., 2010). This is consistent with the observation that *ein2* reduced O_3-induced cell death in the JA-insensitive mutant *jar1* (Tuominen et al., 2004), the extent of O_3-induced cell death was substantially less in *coi1-16 ein2* compared to *coi1-16* (III). The different oxylipins OPDA and JA might have differential roles in the apoplastic ROS signaling, thus *aos* and *aos ein2* were also tested. However, ethylene displayed a similar effect on the regulation of O_3-induced cell death in *aos* as in *coi1-16*. The extent of cell death was substantially reduced in *aos ein2* and *coi1-16 ein2*. (III). In the other mutant combinations, *ein2 sid2* exhibited similar extent of cell death as *ein2* and *sid2*, whereas *coi1-16 ein2 sid2* showed similar amount of cell death as *coi1-16 sid2* and *coi1-16* mutants, suggesting that a basal level of SA could be required for ethylene-dependent suppression of JA signaling in O_3-triggered cell death. Likewise, the inhibitory effect of JA on cell death was also evident in the LMMs *hrl1* (*hypersensitive response-like lesions 1*) and *dnd1* (I)(Devadas et al., 2002). Removal of JA signaling in *hrl1* and *dnd1* enhanced the stunted growth and/or lesion phenotype. Further removal of both SA and JA biosynthesis in the *dnd1 aos sid2* triple mutant resulted in plants that grew better than *dnd1 aos*, but with enhanced cell death (I). The regulation of apoplastic ROS induced cell death and the contribution of SA, JA and ethylene is summarized in Figure 3.

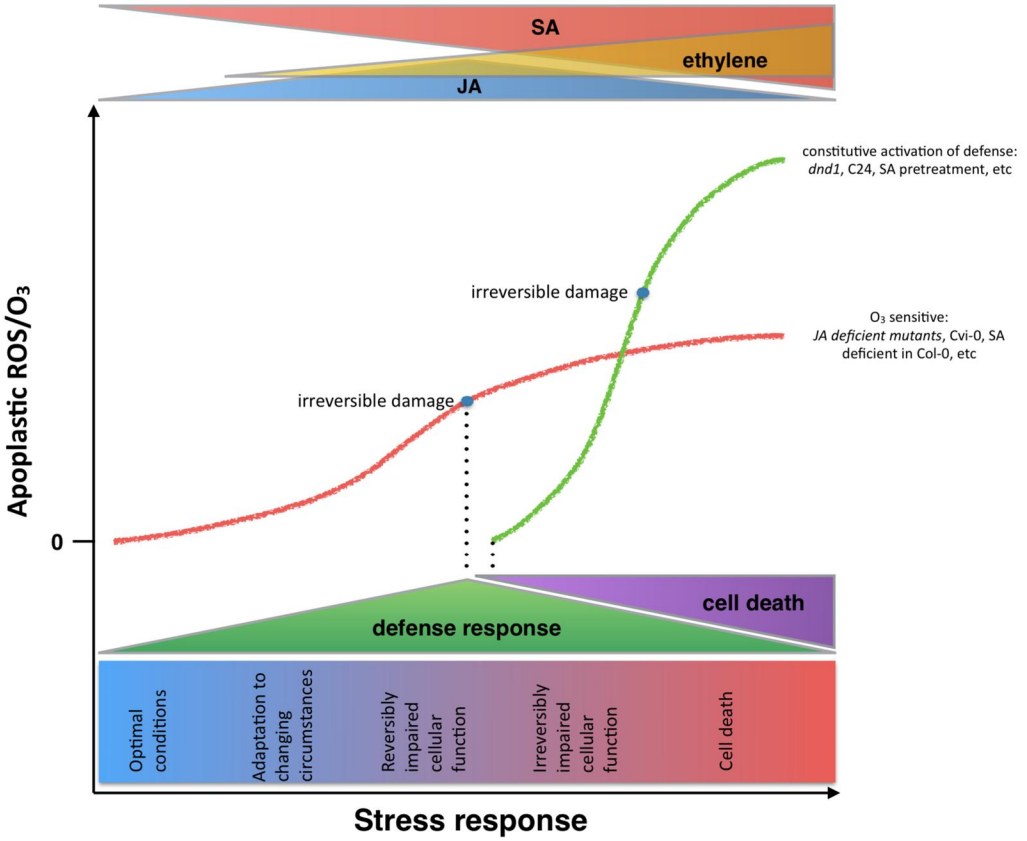

Figure 3. Model for execution of apoplastic ROS mediated defense responses and cell death. In plants under optimal conditions, ROS production is kept under control by various antioxidants. When apoplastic ROS production in plants increase due to moderate stresses, regulation of stress-induced ROS signaling by activation of JA, SA, and ethylene signaling allows plants to activate appropriate defenses and acclimate to the stress. The interaction among these hormones could help plant to prioritize between activation of defenses and initiation of cell death. If the stress persists or higher level of stress occurs, the ROS production may exceed the capacity of the cell to handle, and the balance of defense responses triggered by apoplastic ROS signaling is shifted towards cell death and irreversible damage. The cell death response includes activation of SA and ethylene signaling and suppression of JA signaling. Plants with constitutive activated defenses (i.e. pretreatment with SA or mutants with elevated SA signaling) can occasionally have cell death in the absence of stress, but they also have a raised threshold for when cell death is initiated.

The two closely related regulators *EDS1* and *PAD4* were previously shown to operate upstream of ethylene and ROS production in the response to light stress (Mühlenbock et al., 2008). Furthermore, EDS1 regulates cell death in variety of contexts including LMMs (Rustérucci et al., 2001). The *eds1* mutant was therefore introduced into all *coi1* single, double and triple mutants; surprisingly, O_3-induced cell death in *coi1 eds1*, *coi1 eds1 ein2*, *coi1 eds1 sid2* and *coi1 eds1 ein2 sid2* was not substantially altered compared to the single mutant *coi1-16* (III). Further studies using different O_3 doses and time points might be required to understand the role of EDS1 in O_3-induced cell death.

The *lsd1* mutant is one of the best studied LMMs (Dietrich et al., 1997). Plants lacking both LSD1 and RBOHD or LSD1 and RBOHF exhibited enhanced SA-dependent cell death compared to the *lsd1* single mutant, suggesting that RBOHD and RBOHF-derived ROS could antagonize SA-dependent cell death (Torres et al., 2005). Another LMM is the CATALASE2 deficient *cat2*; in this mutant background RBOHF appears to be required to restrict cell death, since the *cat2 rbohF* double mutant displays increased cell death compared to the single *cat2* mutant (Chaouch et al., 2012). In *dnd1* neither RBOHD nor RBOHF appeared to be important for cell death regulation (I). In O_3-triggered cell death and H_2O_2 accumulation in *coi1-16* the *rbohf* but not *rbohd* mutation led to reduced cell death and H_2O_2 accumulation (III). This suggests that RBOHF could act as a positive regulator in apoplastic ROS-triggered cell death. However, in other mutant background the role for RBOHD and RBOHF appears to be different – thus ROS signaling is very much dependent on the context, including mutant background or stress treatment.

WRKY70 is required for SA-mediated resistance in plant-pathogen interactions and functions as an important regulator of cell death (Li et al., 2004; Besseau et al., 2012). However, no obvious effect on the regulation of cell death was apparent in *dnd1* (I). In contrast, apoplastic ROS-induced cell death was substantially reduced to the level of Col-0 in *coi1 wrky70* (III), suggesting that some specific target genes of WRKY70 might be the crucial regulators of apoplastic ROS-induced cell death.

5 Conclusion and future perspective

Rapid and transient ROS bursts, ion fluxes, diverse hormones changes, and transcriptional reprogramming are known to have central roles in the plant defense system. Apoplastic ROS play an important signaling role during biotic and abiotic stresses, but little is known about how ROS are perceived and how downstream signaling is activated. Arabidopsis offers a convenient system to study apoplastic ROS signaling due to the availability of a huge collection of indexed mutants as well as natural variation in apoplastic ROS-triggered cell death. The natural variation of O_3 responses among different Arabidopsis accessions could allow researchers to find novel genes or alleles involved in apoplastic ROS signaling. In this study, both types of genetic materials were employed to dissect out the genetic basis of responses to apoplastic ROS treatment.

Through QTL mapping in a RIL population, a F2 population and one backcross population, three rather large QTL regions were identified (II). However, identifying the candidate genes underlying the QTL regions is a major challenge in this population due to the accuracy of phenotypic analysis and a lack of high-resolution genetic markers in these populations. As a complementary approach, a RNA-seq analysis was performed to reduce the number of gene candidates (II). Gene expression analysis revealed that constitutively activated SA signaling in the accession C24 could be one explanation for the attenuated O_3 response. This inhibitory effect of SA on the apoplastic ROS responses could be mimicked by pretreatment with exogenous SA (II). Likewise, SA-dependent and independent signaling additively contributed to this SA-inhibitory effect on apoplastic ROS signaling in the O_3-tolerant LMM *dnd1* (I). Combined with previous data showing that SA also can promote cell death, this suggests that SA signaling play a dual role in the regulation of apoplastic ROS signaling and cell death. Although simultaneous blocking of SA-dependent and independent signaling in *dnd1* resulted in

decreased cell death (I), the cell death phenotype was not completely eliminated. Uncovering the bridge between the altered cytosolic Ca^{2+} influx in *dnd1* and downstream signaling pathways could reveal the potential signaling pathways that initiate the defense response. In addition, the primary cause of the constitutively activated defense response in the accession C24 is still elusive. Decoding the genetic basis that confers a broad disease-resistance phenotype without growth penalties in C24 could substantially facilitate future breeding for disease resistant cultivars. Moreover, understanding the interaction between ROS signaling and hormones signaling could shed light on the regulation of defense-growth homeostasis in plants.

JA and ethylene appeared to synergistically and antagonistically participate in the regulation of apoplastic ROS-triggered changes in gene expression and cell death. JA signaling could function to both positively and negatively regulate in some parts of early apoplastic ROS signaling, and JA-triggered signaling was partially enhanced when ethylene signaling was also impaired. However, both constitutively activated and impaired ethylene signaling had on a limited influence on the early-apoplastic ROS signaling (I, III). Intriguingly, transcriptome analysis revealed that a substantial part of apoplastic ROS signaling was very similar between genotypes with different O_3-sensitivities (I, II, III, Figure 2. Possibly, the acute O_3-triggered ROS production exceeds the capacity of the cell for activating a specific defense response. However, the pre-established constitutively activated defenses in *dnd1* or C24, led to a higher threshold for tolerance of a O_3-triggered ROS burst. A model for execution of apoplastic ROS triggered defense response and cell death is proposed in Figure 3. In the future, large-scale transcriptome analysis using various mutants, doses and a time-course of apoplastic ROS treatments would explain how the induction of gene expression could lead to either survival or death. Furthermore such analysis might uncover how plants can activate and prioritize between potentially conflicting defense signaling pathways, and reveal how the dynamic balance between defense and growth is maintained.

Summary in Finnish

Solutason homeostaasin säätely on tärkeää yksilönkehityksen, puolustusvasteiden, ohjelmoidun solukuoleman ja lopulta selviytymisen kannalta. Homeostaasin säilyttäminen vaatii useiden keskenään vuorovaikutteisten viestinvälitysreittien tarkkaa säätelyä. Apoplasti sijaitsee solun ja sen ympäristön välisellä rajalla, josta kasvi aistii ympäristön muutoksia. Lisäksi apoplastin kautta kulkee osa solujen välisestä viestinnästä, joten sillä on tärkeitä tehtäviä kasvin ja sen ympäristön välisessä vuorovaikutuksessa. Reaktiiviset happilajit (reactive oxygen species, ROS) tunnetaan sekä myrkyllisinä yhdisteinä että korvaamattomina viestintämolekyyleinä kaikissa happea tarvitsevissa eliöissä. Yksi ensimmäisistä havaittavista vasteista erilaisiin bioottisiin ja abioottisiin stresseihin on apoplastissa tapahtuva reaktiivisten happilajien purkaus (ROS-purkaus), joka johtaa viestinvälitysreittien aktivaatioon ja muutoksiin geenien ilmentymisessä. Apoplastissa tapahtuva ROS-viestintä koordinoi dynaamisesti useita viestinvälitysketjuja kasvin puolustusvasteiden aktivoinnin aikana. Viestinvälitysketjujen ristivaikutusten analyysi saattaa siten paljastaa puolustusvasteen molekyylimekanismeja. Kasvien käsittelyä otsonilla (O3) käytetään tehokkaana työkaluna apoplastisen ROS-viestinnän tutkimuksessa. Kasvin otsonikäsittely laukaisee apoplastissa ROS-purkauksen ja aikaansaa suuria muutoksia geenien ilmentymisessä ja puolustukseen liittyvissä hormoneissa, kuten salisyylihapossa, jasmiinihapossa ja etyleenissä.

Lituruohon (Arabidopsis thaliana) perinnöllinen muuntelu otsoniherkkyydessä korostaa kasvien ROS-vasteiden monimutkaista geneettistä rakennetta. Tässä työssä apoplastisen ROS-viestinnän geneettistä taustaa selvitettiin kvantitatiivisiin ominaisuuksiin vaikuttavien lokusten (QTL) kartoittamisella kahden Arabidopsis-ekotyypin, C24 (otsonikestävä) ja Tenela (otsoniherkkä), välisessä risteytysjälkeläistössä. Yhdistelemällä apoplastisen ROS-käsittelyn vaikutuksen QTL-kartoitusta ja transkriptomin tutkimusta löydettiin kolme QTL-aluetta, joissa on useita kandidaattigeenejä. Apoplastisen ROS-viestinnän ja otsonin käynnistämän solukuoleman parempaa ymmärtämistä varten tutkittiin lisäksi useita mutanttilinjoja, jotka eroavat toisistaan otsoniherkyydeltään. Laajaa ja kohdistettua geenien ilmentymisen profilointia, geneettistä analyysiä ja solukuolemakokeita käytettiin erittelemään hormoniviestinnän ja useiden transkriptiotekijöiden vaikutusta reaktiivisten happilajien käynnistämään apoplastiseen geenien ilmenemiseen ja solukuolemaan.

Salisyylihapon, jasmiinihapon ja etyleenin tehtäviä tutkittiin analysoimalla mutanttilinjoja, joilta kyseisten hormonien viestintä oli estetty tai tehostettu, sekä lisäämällä hormoneja kasveille ulkoisesti. Kasvien, joilla oli korkeammat salisyylihappotasot, huomattiin reagoivan otsoniin vaimeammin, kun taas samanaikainen salisyylihaposta riippuvan ja riippumattoman viestinnän estäminen voimisti vastetta apoplastiseen ROS-käsittelyyn. Jasmiinihappo toimi sekä positiivisena että negatiivisena säätelijänä apoplastisessa ROS-viestinnässä, ja sen toiminta tehostui, kun myös etyleeniviestintä oli vaimennettu. Toisaalta kolmoismutantin, jolta oli vaimennettu kaikkien kolmen edellä mainitun hormonin viestintä, transkriptomitason analyysi paljasti, että näiden hormonien kautta kulkeva viestintä vaikuttaa vain osaan (noin 30%) varhaisista geenien ilmentymisen muutoksista vasteena apoplastiseen ROS-viestintään. Tästä voidaan päätellä, että useat viestinvälitysketjut säätelevät apoplastista ROS-vastetta päällekkäisin mekanismein.

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